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Identification and Characterization
of SNO Regulated Genes (*SRGs*) in plant
immunity

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Abstract

A conspicuous feature of plants responding to pathogen invasion is the synthesis of nitric oxide (NO), a redox signal. NO regulates protein function by S-nitrosylation, the addition of an NO moiety to a cysteine thiol to form an S-nitrosothiol. A key theme of NO function is reprogramming plant immune-related gene expression. However, it is still not clear how the NO signal is translated into transcriptional changes. Here we explored the potential role of a sub-group of SNO Regulated Genes (*SRGs*) uncovered by global expression profiling.

Firstly, transgenic plants containing the *SRG1* or *SRG3* promoter fused to glucuronidase gene *GUS* together with qRT-PCR assays confirmed that transcripts of *SRGs* could be induced by NO and pathogen challenge, suggesting that *SRGs* may be involved in NO signalling related to plant immunity. More importantly, transient and stable overexpression of *SRG* genes induced hypersensitive response (HR)-like cell death development, which is often associated with pathogen effector-triggered immunity. Furthermore, transgenic plants constitutively expressing *SRG* genes exhibited enhanced ROS accumulation, *PR1* transcript accumulation, and increased resistance to *Pseudomonas syringae* (*Pst*) DC3000 compared with Col-0 wild type plants. In contrast, lines with T-DNA insertions into *SRG* genes exhibited susceptibility to *Pst* DC3000. These data suggested *SRGs* act as the positive regulators in plant immunity.

In order to further explore how NO regulates these *SRGs* in plant immunity, we focused on *SRG1* and found *SRG1* could be S-nitrosylated *in vitro* and *in vivo*. Moreover, electrophoretic mobility shift assays showed *SRG1* could bind to an AGT motif and the transcriptional activity was blunted in the presence of NO, suggesting that the DNA binding activity of *SRG1* is redox-modulated. Further, a transient repression activity assay showed that *SRG1* has repression activity and this activity was impaired in the *gsnor1-3* mutant, which has a high S-nitrosothiols level. These data suggested NO could block *SRG1* transcriptional activity *in vitro* and *in vivo*. Furthermore when the *SRG1* overexpression line was crossed with *gsnor1-3* the *SRG1*-mediated resistance related phenotypes were suppressed. These data demonstrated NO negatively regulates *SRG1* transcriptional activity during plant

immunity.

SRG1 may therefore be an important regulator of NO signalling and subsequent regulate transcription during plant immunity. Additionally, NO may negatively feedback to inhibit transcriptional activity of SRG1 to control its repression activity, to enable the activation of plant immunity.

Lay Summary

A conspicuous feature of plants responding to pathogen invasion is the synthesis of nitric oxide (NO) and a key theme of NO function is reprogramming plant immune-related gene expression. However, it is still not clear how the NO signal is translated into transcriptional changes. Here we explored the potential roles of a sub-group of SNO Regulated Genes (*SRGs*) uncovered by global expression profiling. *SRGs* encoded C2H2 type Zinc Finger (ZF) transcription factors and transgenic plants constitutively expressing *SRG* genes exhibited enhanced ROS accumulation, *PR1* transcript accumulation, and increased resistance to *Pseudomonas syringae*, whereas *srg* mutant exhibited susceptibility to *Pst* DC3000. Interestingly, SRG1 could be S-nitrosylated *in vitro/vivo*, resulting in inhibiting its transcriptional activity. Moreover, when the *SRG1* overexpression line was crossed with *gsnor1-3* the *SRG1*-mediated resistance related phenotypes were suppressed. These data demonstrated SRG1 may therefore be an NO sensor and subsequent regulator of transcription during plant immunity.

Declaration

I hereby declare that the work presented here is my own except where explicitly stated in the text* and has not been submitted in any form for any degree at this or other university.

Beimi Cui

*Data of qRT-PCR, bacterial growth assay and confocal microscope observations shown in **Figure 3.7, 3.8, 3.11, 4.12, 4.14, 5.2, 5.5, 5.8, 5.10, 6.4 and 6.5** were co-worked with Qiaona Pan and permission has been gained from Qiaona Pan for inclusion in this thesis.

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List of abbreviations

Abbreviation	Full name
35S/CaMV 35S	Cauliflower Mosaic Virus 35S promoter
3-AT	3-amino-1,2,4-triazole
5'UTR	5' Untranslated Region
AD	Activation Domain
ATP	Adenosine triphosphate
BD	Binding domain
BiFC	Bimolecular fluorescence complementation
bp	basepair
CDS	Coding sequences
cDNA	complementary DNA
cPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CTAB	Cetyltrimethylammonium bromide
Cys	Cystein
Cys-NO	S-nitrosocysteine
DAB	3,3'-Diaminobenzidine
DADPH	Nicotinamide adenine dinucleotide phosphate
DAPI	4', 6-diamidino-2-phenylindole
dpi	day(s) post inoculation
DTT	Dithiothreitol
EAR	Ethylene-responsive element binding factor-associated Amphiphilic Repression
<i>edr1</i>	<i>enhanced disease resistance 1</i>
EMSA	Electrophoretic mobility shift assay
FAD	Flavin Adenine Dinucleotide
gDNA	genomic DNA
GFP	Green fluorescent protein
GSNO	S-Nitrosoglutathione
GSNOR	S-Nitrosoglutathione reductase
GST	Glutathione S-transferase
GUS	β -glucuronidase
hpi	hour(s) post inoculation

HR	Hypersensitive Response
IB	Immunoblotting
IP	Immunoprecipitation
IPTG	isopropyl β -D-1-thiogalactopyranoside
kb	kilobases
kDa	kilodaltons
LC-MS	Liquid chromatography-mass spectrometry
LUC	Luciferase
MAPK	Mitogen-activated protein kinases
MBP	Maltose binding protein
MEF2	Myocyte enhancer factor 2
MG132	Carbobenzoxy-Leu-Leu-leucinal
μ M	micromolar
mM	millimolar
mRNA	Messenger RNA
MS	Murashige and Skoog medium
NASC	The Nottingham Arabidopsis Stock Centre
NBT	Nitroblue tetrazolium
NINJA	Novel interactor of JAZ
NLS	Nuclear localization signal or sequence
NO	Nitric Oxide
NPR1	Nonexpressor of pathogenesis-related genes 1
OX	Overexpression
P.S	Ponceau Stain
<i>pad4</i>	<i>phytoalexin deficient 4</i>
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PR1	Pathogenesis-related gene 1
<i>Pst</i> DC3000	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000
PTM	Posttranslational modification
qRT-PCR	quantitative Reverse transcription polymerase chain reaction
RBOHD	Respiratory burst oxidase-D
<i>R</i> -gene	Resistance gene

ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic acid
SABP3	SA-binding protein 3
SD	Synthetic drop out medium
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNO	S-nitrosothiol
SNP	Sodium nitroprusside
T-DNA	Transfer DNA
TGA1	TGACG Sequence-specific binding protein 1
UBQ10	Polyubiquitin 10
X-alpha-gal	5-bromo-4-chloro-3-indolyl-alpha-D-galactopyranoside
Y2H	Yeast two-hybrid
YC	YFP-C-terminus
YFP	Yellow fluorescent protein
YN	YFP-N-terminus

Chapter

1 Introduction

1.1 General introduction

In nature, plants are constantly exposed to various environmental stresses, such as low/high temperature, drought, salinity, as well as pathogen infection. In order to successfully defend themselves, plants have developed a series of defence mechanisms including physical barriers and inducible response to combat different types of stresses ([Kliebenstein, 2012](#)). For example, upon pathogen infection, plants have evolved a two-branched system of innate immunity ([Jones and Dangl, 2006](#)). Plant hormones also play essential roles in immune responses, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) ([Pieterse et al., 2009](#)). According to their lifestyle, pathogens are divided into necrotrophs and biotrophs ([Lenz et al., 2011](#)). Necrotrophic pathogens normally destroy the host cell and take nutrients from dead cells, while biotrophic pathogens derive nutrients from living tissues. In order to better understand plant immunity, efforts have to be made for identification of the elements that are involved in plant defence.

1.2 Plant defence system

1.2.1 Plant pre-formed immunity

In order to prevent microbial invasion, plants have established a variety of pre-formed barriers and chemicals as broad-spectrum defences ([Heath, 2000](#)), for example, waxy cuticles ([Yeats and Rose, 2013](#)), lignin and poly-saccharides ([Bednarek and Osbourn, 2009](#)). Wax biosynthetic gene was induced in the presence of pathogens ([Yeats and Rose, 2013](#)). In addition to physical barriers, plants produce a diverse array of antimicrobial metabolites for defence ([Bednarek and Osbourn, 2009](#)), including phytoanticipins ([Dixon, 2001](#)). Apart from these preformed chemicals, others can be produced in response to pathogen challenging, for example phytoalexins ([Dixon, 2001](#)). In addition to those antimicrobial chemicals, there are also secondary metabolites, antimicrobial proteins, which have antimicrobial activity ([Bednarek and](#)

[Osbourn, 2009](#)). Increasing evidences showed plant antimicrobial peptides could inhibit pathogen growth and colonization ([Maróti et al., 2011](#)). All of these mechanisms are preformed in response to pathogen challenging and inhibit pathogen entry into host cells.

1.2.2 Plant immunity

Plants have developed a highly sophisticated immunity system ([Wit, 2007](#), [Ellis et al., 2009](#)). There are two branches of basal immunity in plants ([Jones and Dangl, 2006](#)). As shown in figure 1.1, the first branch is perception and recognition of non-self molecules, which are called pathogen-associated molecular patterns (termed as PAMPs), by pattern recognition receptors (PRRs) located in the plasma membrane. After recognition, PRRs can transduce the signal into cells and then result in PAMP-triggered immunity, which is termed as PTI. For example, FLS2 ([Wit, 2007](#)) and EFR ([Zipfel et al., 2006](#)) are conserved PRRs in membrane which could recognize the PAMP flagellin and elongation factor Tu (EF-Tu), respectively. Evolutionally, pathogen could crossover this defence by secreting effectors that could repress PTI and lead to colonization (termed as Effector-triggered susceptibility, ETS). In turn, plants evolved the resistance protein (R protein, encoded by *Resistance* gene, *R* gene). R proteins could recognize corresponding effector and initiate defence responses, and this process is called effector-triggered immunity (ETI), resulting in reactive oxygen species (ROS) burst and hypersensitive response (HR).

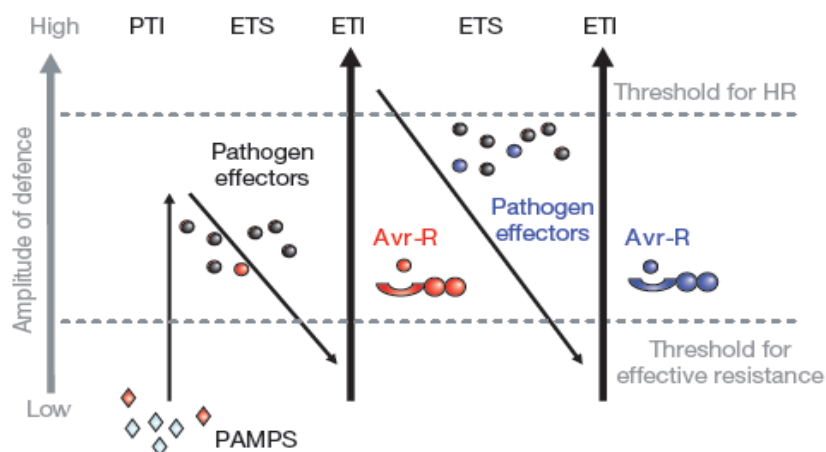


Figure 1.1 Zig-zag model of plant immunity.

Firstly, detection of pathogen-associated molecular patterns (PAMPS) by pattern recognition receptors (PRRs) triggered PAMP triggered immunity (PTI). Secondly, pathogens overcome PTI through secretion of effectors to interfere with PTI. Thirdly, some effectors can be recognized by the corresponding R protein, effector triggered immunity is engaged. Adapted from Jones and Dangl ([2006](#)).

1.2.2.1 PAMP-triggered immunity (PTI)

In higher plants, PTI is the basal defence response, and plays important functions in immunity. It is demonstrated that application of synthesized conserved-peptide EF-Tu ([Zipfel et al., 2006](#)) or Flg22 ([Chinchilla et al., 2007](#)) in *Arabidopsis* induced defence genes expression, suggesting these PAMPs could be recognized by plant PRRs. In this process, recognition of a PAMP by PRR is the most crucial process and current research mainly focuses on the interactions between PAMPs and PRRs upon pathogen infection ([Chinchilla et al., 2007](#), [Macho and Zipfel, 2014](#)). By forward genetic screening, some PRRs in host plants were identified and characterized, such as FLS2 ([Gómez-Gómez et al., 2001](#), [Zipfel et al., 2004](#)). Using molecular technologies, the co-receptors in PTI were found, for example BAK1 ([Heese et al., 2007](#), [Chinchilla et al., 2009](#)) and BIK1 ([Veronese et al., 2006](#), [Lu et al., 2010](#)). Furthermore, recently crystal structures uncovered the mechanism of interactions between FLS2 and BAK1 in the presence of flagellin and the activation of downstream signalling ([Sun et al., 2013](#)). This provided direct evidence to support the idea that the plant PRR, FLS2, recruits its co-receptor BAK1 to trigger plant defence response in the presence of flg22. The mechanisms of immune responses are very complex and more regulators of PTI likely remain to be identified. Specifically, as shown in figure 1.2 the *Arabidopsis* PRR FLS2 could recognize the conserved N-terminal 22 amino acids of bacterial PAMP flagellin and then recruit the receptor BAK1 (BRI1-associated receptor kinase 1). After that BIK1 (BOTRYTIS-INDUCED KINASE 1), a substrate of BAK1, is phosphorylated by BAK1 and then initialises defence responses by activating the downstream signalling, such as the ROS burst ([Yoshioka et al., 2009](#)) and mitogen-activated protein kinases (MAPK) pathways ([Hamel et al., 2012](#)). In turn, plants have negative regulators to control PTI (Figure 1.2). A small receptor like kinase BIR1 (BAK1-interacting receptor-like kinase 1) was

identified to interact with BAK1 and negatively regulate plant defence responses ([Gao et al., 2009](#)). Besides, BIR2, a novel BAK1-interacting receptor kinase, negatively regulates PTI by preventing the interaction between BAK1 and FLS2 in response to PAMP ([Halter et al., 2014](#)). In addition, another repressor PP2A, a protein Ser/Thr phosphatase type 2A, could interrupt the phosphorylation of BAK1, which is required for receptor-ligand interaction in PTI ([Segonzac et al., 2014](#)). Furthermore, a Ca^{2+} -dependent protein kinase CPK28 was recently found to act as a repressor of BIK1 ([Monaghan et al., 2014](#)). In particular, the CPK28 interacts with and phosphorylates receptor BIK1, resulting in turning over of BIK1 and compromising PTI signalling. Genetic analysis also showed *cpk28* mutants could promote PTI, whereas overexpressing *CPK28* attenuated PTI signalling. Overall, PRRs play importance roles in plant immunity and negative regulators could control PTI (Figure1.2).

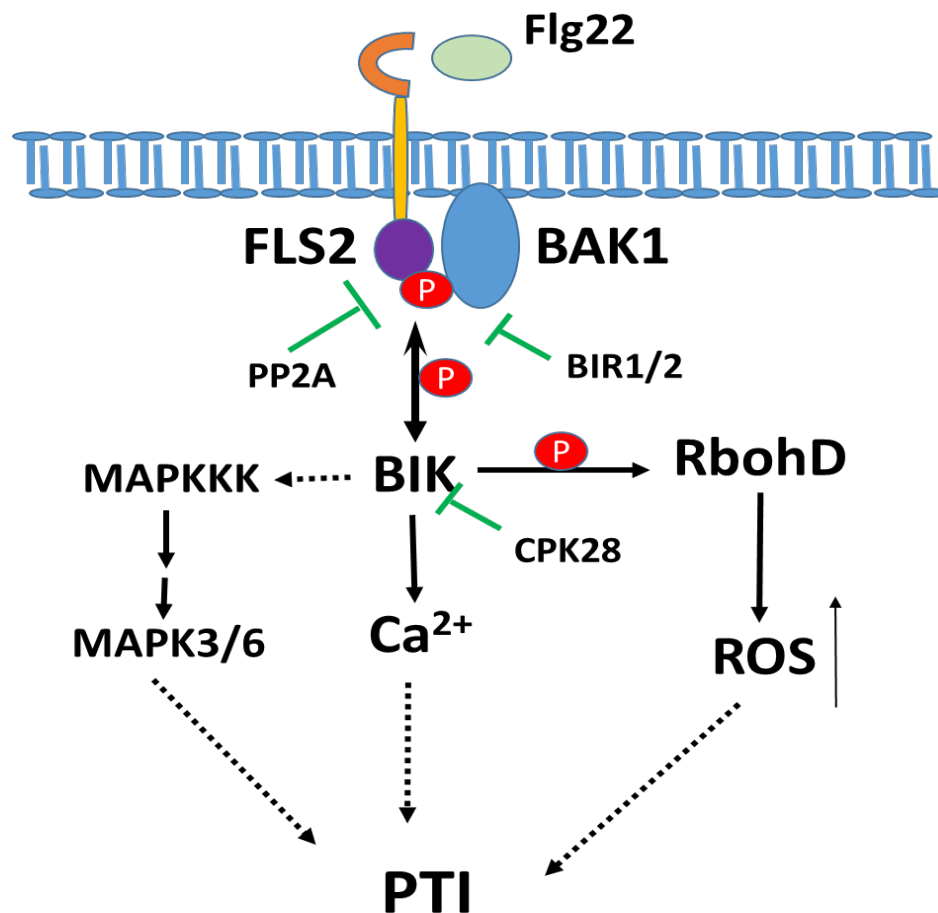



Figure 1.2 Regulation of PAMP-triggered signalling in plants.

After recognition of non-self molecule PAMPs, such as flg22, a pattern recognition receptor (e. g. BAK1) can interact with a co-receptor (e. g. BIK1) and then activate each other by phosphorylation. Then activation of receptors leads to initiate the downstream signalling, such as MAPKs and ROS burst, to activate PTI. However, PTI can be suppressed by negative regulators in plants (e.g. PP2A or CPK28). Black arrows indicate positive interactions. Blocked arrows (blue) represent the repression of signalling pathway. Full lines represent the known or potential regulator. Dotted lines represent more than one step or indirect interactions.  represents phosphorylation process.

1.2.2.2 Effector-triggered susceptibility (ETS)

Upon recognition of PAMPs, plants can activate PTI to limit microbial colonization. However, pathogens produce effectors to interfere with PTI, resulting in overcoming this innate immune response, and successfully causing disease ([Macho and Zipfel, 2015](#)). In order to deliver the effector to host cells, bacterial pathogens have developed a type III secretion system (T3SS), which assembles a molecular syringe to deliver effectors into host cells ([Galan and Wolf-Watz, 2006](#), [Hauser, 2009](#)). The mechanism of effectors to overcome PTI depends on their capacity to repress plant immunity or enable pathogen spread (Jones and Dangl 2006).

To repress plant PTI, pathogens evolve effectors to attenuate PTI by targeting these receptors or their co-receptors ([Macho and Zipfel, 2015](#)). Increasing lines of examples proposed that these effectors could interact with and inhibit PRRs by inhibiting their kinase activity or stability (Figure 1.3). A typical example is effector AvrPto from *Pseudomonas syringae* (*P. syringae*) which could block PTI signalling by inhibiting PRRs FLS2 and EFR kinase activity ([Shan et al., 2008](#), [Xiang et al., 2008](#)). Notably, AvrPto interacts with FLS2 /EFR *in vivo* and results in loss of kinase activity in FLS and EFR, which is essential for PTI signalling. In addition, several effectors from bacteria also exhibited the ability to promote the target receptors degradation by the proteasome. For instance, AvrPtoB has a conserved E3 ubiquitin ligase function and facilitates FLS2 degradation by the proteasome system, leading to a diminish PTI response ([Göhre et al., 2008](#)). Further, AvrPto could physically interact with PRR co-receptor BAK1 to intercept the complex forming of FLS2 and

BAK1 in the presence of pathogen, *P.seudonomas* (Shan et al., 2008). Furthermore, effectors not only interrupt the PRRs and their co-receptors, but also target proteins downstream of PTI, such as those implicated in MAPK cascades. Effectors are proposed to inactivate the kinase activity of MAPKs to compromise PTI signalling. The *Arabidopsis* MAPK3 and MAPK6 were directly targeted by the effector HopA1, a conserved effector in bacterial pathogens, *Pseudomonas* (Zhang et al., 2007). Significantly, HopA1 is a phosphothreonine lyase and it displays inhibition of MAPKs activity by dephosphorylation, which blunts PTI signalling. As shown in figure 1.3, some other effectors have been identified to facilitate pathogen spread by inhibiting PTI (Zhang et al., 2007). Therefore, it is apparent that pathogens could recognize and target multiple proteins in PTI and their downstream pathways.

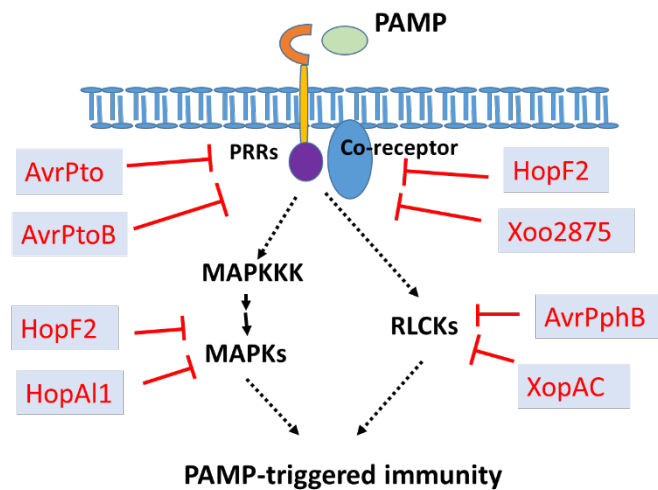


Figure 1.3 Bacterial effectors can inhibit PTI by suppression of receptors and downstream signalling pathways.

The components of PTI can be the target of pathogen effectors, resulting in suppression of PTI, leading to susceptibility. More details were described in context. Adapted from Macho and Zipfel (2015).

1.2.2.3 Effector-triggered immunity (ETI)

Evolutionally, adapted host plants can recognize pathogen effectors by host proteins encoded by *R* genes, leading to ETI (Jones and Dangl, 2006). Most products of *R* genes are Nucleotide Binding-Leucine Rich Repeat (NB-LRR) proteins and ~150 potential *R* genes were predicted in *Arabidopsis* genome (Meyers et al., 2003). ETI is

proposed as the second layer defence response and it is stronger and faster than PTI. Thereby ETI is recognized as an enhanced version of PTI as both of them activate the same downstream response, such as ROS burst and MAPK cascades. Generally, as shown in figure 1.4, recognition of an effector by a R protein restricts pathogen proliferation ([Lam, 2004](#)). Generally, an effector from a pathogen can be recognized by the corresponding NB-LRR or NB-LRR like protein and the effector is then termed an avirulence (Avr) protein, meanwhile the NB-LRR or NB-LRR like protein is termed as an R protein ([Eitas and Dangl, 2010](#)).

The interaction between Avr proteins and R proteins has been a hot research theme for a long time. For instance, *Arabidopsis* RPM1 is a plasma membrane localized NB-LRR protein that can recognize the effector AvrB and AvrRpm1 and then induce immune responses ([Bisgrove et al., 1994](#)). In addition, several pairs of Avr-R were identified to understand plant ETI, such as RPS4 and avrRps4 ([Eitas and Dangl, 2010](#)). Molecular biology assays showed that direct interaction between effector and R Protein is required to trigger ETI ([Jia et al., 2000](#)). Meanwhile, indirect recognition of effectors by corresponding R proteins were also observed ([DeYoung and Innes, 2006](#)). Moreover, activation of immune responses by effector recognition is crucial and is associated with HR and ROS. For instance, RPM1 was reported to recognize AvrRpm1 and resulted in localized HR to limit *P. syringae* proliferation, whereas the *rpm1* mutants showed susceptibility to *P. syringae*, suggesting the interaction between effector and the corresponding R protein is required for plant defences ([Grant et al., 1995](#), [Boyes et al., 1998](#), [Mackey et al., 2002](#)). To date, the mechanism of ETI is not fully understood. Thus, identification of more effectors and regulators in ETI are required to understand the mechanism of pathogen recognition, as we can fine the evolutionary relationship between host and pathogens and it will provide more evidence for new insight for ETI.

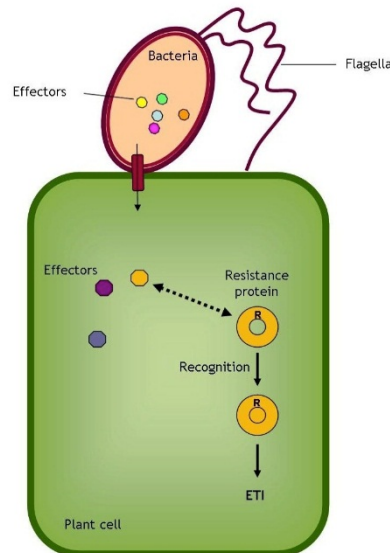


Figure 1.4 Recognition of effectors by host corresponding resistance proteins.

Effectors secreted by plant pathogens can be recognised by host R proteins, resulting in effector-triggered immunity (ETI). More details were described in context.

Source: http://www.pseudomonas-syringae.org/Outreach/Module_4_Lab.htm

1.3 Plant defence signalling

1.3.1 Salicylic acid signalling in plant immunity

Plant hormones are small molecules that play pivotal roles in the regulation of immunity responses and function at a low concentration (Pieterse et al., 2009). An important plant hormone for plant disease resistance is salicylic acid (SA), which is specific against biotrophic pathogens. Research has shown that SA functions after PTI or ETI, because SA synthesis is induced during PTI and ETI (Pieterse et al., 2012). As a secondary signalling molecule, Ca^{2+} was shown to function in the upstream of SA signalling, and upon pathogen infection Ca^{2+} levels are increased and then SA biosynthesis is triggered (Boursiac et al., 2010). In addition, Protein enhanced disease susceptibility 1 (EDS1) and phytoalexin deficient 4 (PAD4) work together to induce SA pathway (Wiermer et al., 2005). *NPR1* (nonexpressor of pathogenesis-related gene 1) is the most important component in the downstream of SA signalling (Dong, 2004, Tada et al., 2008). After SA increase in plant cell, *NPR1* activates the pathogenesis-related genes (*PRs*), some of which encode antimicrobial protein and

then enhance the plant disease resistance. For example, *PR1*, *PR2* and *PR5* are used as the SA-responsive marker genes. In native cell, NPR1, a master protein for controlling *PR* genes expression, mainly locates in cytosol as the form of oligomer by intermolecular disulfide bonds between cysteine residues. However, upon pathogen challenging, the level of SA increases and subsequently changes the redox states which leads to induce the form of NPR1 from oligomers to monomers, then NPR1 monomers move to nucleus, where it can activate *PR* genes expression and initiate plant defence response. Plants have also evolved a mechanism to control the level of nuclear NPR1. Nuclear translocated NPR1 interacts with transcriptional factors, TGA proteins, a subclass of the basic leucine zipper (bZIP) family, which recognize and bind to the promoters of SA responsive genes, such as *PR* genes, subsequently activating SA-mediated plant defence ([Lindermayr et al., 2010](#)). In addition, its paralogues NPR3 and NPR4 are also involved in SA-mediated signalling by regulating the balance of NPR1. Specifically, NRP3 and NRP4 could recruit Cullin 3, an E3 ligase, to promote the degradation of NPR1 and keep the plant fitness ([Fu et al., 2012](#)).

1.3.2 Reactive oxygen species signalling in plant immunity

Reactive oxygen species (ROS) are forms of reactive molecular oxygen including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radical (HO^{\cdot}). ROS is regarded as a rapid and important second messenger in plant signal transduction which modulates cellular redox status. During plant abiotic or biotic stress, production of ROS is a key feature to modulate adaption to stresses ([Mittler et al., 2004](#)). For example, ROS production at the pathogen infection site induced cell death, which could prevent pathogen spread ([Govrin and Levine, 2000](#)). ROS is also scavenged after plants are removed from stress conditions to avoid damage to plant cell ([Asada, 2006](#)). During pathogen challenging, the rapid accumulation of ROS is one of the most important defence reactions and this process we called oxidative burst. ROS scavenging enzymes ascorbate peroxidase (APX) and catalase (CAT) are blocked by SA and Nitric Oxide (NO), which is another way to maintain a high level of ROS ([Klessig et al., 2000](#)). Pathogen infection triggers ROS production, via enzymes such as cell wall peroxidases and NADPH oxidases ([Grant and Loake, 2000](#),

[Spoel and Loake, 2011](#)). Genetic analysis of *Arabidopsis* treated with fungal cell wall elicitor showed PEROXIDASE 33 is a major contributor to the ROS burst ([Daudi et al., 2012](#)). In addition to PEROXIDASE, membrane-bound NADPH oxidases, also called respiratory burst oxidases (RBOHs), is an important enzyme for ROS synthesis. Production of ROS resulting from pathogen infection led to changes of the redox environment, which regulates gene expression, protein activity and stability ([Mersmann et al., 2010](#)). More importantly, ROS function as a second molecular messenger to activate downstream pathways and amplify signals. For example, H₂O₂ is the major form of ROS in plant defence and it is capable of diffusing and activating many plant defences, especially programmed cell death (PCD) ([Gechev et al., 2006](#)). In plant there are ROS sensors, which can sense and convert ROS signalling to downstream targets, such as MAPKs and transcription factors. For example, in *Arabidopsis* application of H₂O₂ increases the activity of MAPK3 and MAPK6, two of the most important components in plant immunity, through activation of MAPKKK ([Takahashi et al., 2011](#)). ROS also associate with SA and regulate plant systemic immunity ([Vlot et al., 2009](#)).

1.3.3 Role of MAPK signalling in plant immunity

In plants MAPK pathways are pivotal in signal transduction from extracellular stimuli and amplifying the signal via cascades, which include three layers functional protein kinases (MAPK kinase kinase, MAPK kinase and MAPK). More evidence showed MAPKs are early signals in the response to pathogen infection ([He, 2006](#), [Meng and Zhang, 2013](#)). During pathogen infection, MAPKs are activated and then modify their target protein by phosphorylation, resulting in the switch on or off of the defence pathway. In responses to different types of pathogens, different MAPKs are activated in plants. For example, inoculation with the oomycete pathogen *phytophthora infestans* induces expression of *MAP3K19* and *MAP2K4*, while infection with *Botrytis cinerea* results in expression of *MAP3K18* and *MAP3K20* ([Menges et al., 2008](#)). There are two layers in plant innate immunity, ETI and PTI, both of which involve MAPKs. For example, the bacterial flagellin receptor FLS2 triggers *MAPK* expression after recognition of flg22 ([Ichimura et al., 2006](#)). After activation, MAPK cascades regulate plant disease resistance by modulating ROS

production, hormone biosynthesis, defence gene expression and the activity of transcription factors ([Asai et al., 2002](#), [Menges et al., 2008](#), [Meng and Zhang, 2013](#)). Publications have shown expression of MAPK3/6 induced ROS production, while inactivation of MAPK3/6 decreased ROS accumulation ([Mittler et al., 2004](#)). MAPKs can also modulate enzymes for the biosynthesis of hormones by phosphorylation, such as ACS2/ACS ([Liu and Zhang, 2004](#)). More importantly, MAPKs regulate signal transduction by recognition of substrates and their subsequent modification via phosphorylation ([Meng et al., 2013](#)). Many substrates are transcription factors, such as WRKY33([Qiu et al., 2008](#)), zinc Finger proteins ([Hamel et al., 2011](#)), and proteins that function as positive or negative regulators of some defence genes. For instance, MAPK6 phosphorylates ERF104 directly, which then leads to an increase in the expression of the defence gene *PDF1.2a* in the presence of flg22 ([Bethke et al., 2009](#)). Transcription factor WRKY33 is required for MAPK3/6 to induce accumulation of the antimicrobial compound camalexin and it can bind to its own promoter to regulate itself ([Zheng et al., 2006](#)). A recent study showed *Botrytis cinerea* induced phosphorylation of WRKY33 by MAPK3/6 and then lead to increase camalexin accumulation. Without phosphorylation of WRKY33 by MAPK3 and MAPK6, no camalexin is produced ([Zheng et al., 2006](#), [Mao et al., 2011](#), [Birkenbihl et al., 2012](#)).

1.4 Nitric Oxide and disease resistance

As a gaseous biological signalling molecule, Nitric Oxide (NO) exhibits a variety of roles in plants ([Bellin et al., 2013](#)). Especially in the last 30 years, varied functions of NO have been identified and attracted great attention. Because of its important roles, NO was termed ‘Molecule of the Year’ by *Science* journal in 1992 and the work on NO in mammals was the subject of the Noble prize in 1998 ([Yu et al., 2014](#)). In plants, NO can also mediate multiple processes, including development and stress responses and increasing evidences show NO is a key plant defence signal molecule ([Wendehenne et al., 2004](#)).

1.4.1 Biosynthesis of NO in plants

In animals, the production of NO is primarily generated by nitric oxide synthase (NOS) enzymes, which are capable of catalysing the conversion of L-Arginine to

L-Citrulline and NO ([Wienerroither et al., 2014](#)). To date, there are no homologous *NOS* genes or NOS-like proteins identified in higher plants ([Wendehenne et al., 2004](#)). Despite this, several loss-of-function mutants provide knowledge about NO biosynthesis in plants. It is worth pointing that the *Arabidopsis* mutant *atnos1* was unable to accumulate NO ([Guo et al., 2003](#)). *AtNOS* has been shown to encode a GTPase rather than a NOS-like protein and therefore if this protein is involved in NO synthesis its role is likely to be indirect ([Zemojtel et al., 2006](#)). Further, a loss-of-function mutation in *CUE1* (*CHLOROPHYLL A/B BINDING PROTEIN UNDEREXPRESSED1*) / *Nitric Oxide-Overproducing 1* (*NOX1*), exhibits constitutive overproduction NO ([He et al., 2004](#)). *CUE1/NOX1* encodes a plastid inner envelope phosphoenolpyruvate (PEP)/phosphate translocator (PPT) ([Li et al., 1995](#), [Streatfield et al., 1999](#)). Interestingly, in relation to wild-type, *nox1* mutants increase the content of L-Arginine, the precursor of NO in animals ([He et al., 2004](#)). These results indicate that NO biosynthesis in plants may occur through a NOS-like enzyme. Recent research also suggested that *Arabidopsis* may have metabolic pathway to produce NO resulting from L-Arginine ([Frunzillo et al., 2014](#)).

Another possible protein integral to NO production is Nitrate reductase (NR), which may reduce nitrite to NO ([Hu et al., 2014](#), [Lu et al., 2014](#), [Jian et al., 2015](#)). Although NR in plants might be relevant for NO production, it is unlikely it is the major enzyme to mediate NO synthesis. This is because only 1% of total NR reduction activity is used for NO production and this reaction is dependent on a high concentration of nitrite ([Rockel et al., 2002](#), [Lu et al., 2014](#)). Collectively, these results highlight that the biosynthesis of NO in plants is very complex and likely there are multiple sources to synthesis NO.

1.4.2 NO in plant immunity

As a key redox signalling molecule, NO plays pivotal functions in plant development and stress responses ([Wendehenne et al., 2004](#), [Arasimowicz and Floryszak-Wieczorek, 2007](#), [Bellin et al., 2013](#), [Yu et al., 2014](#)). In response to bacterial pathogens, the NO burst is a conspicuous feature ([Feechan et al., 2005](#)). Specifically, research showed that pathogen infection induces the production of NO in plants and NO scavengers promote bacterial growth in plant leaf tissues ([Delledonne](#)

[et al., 1998](#)). In *Arabidopsis AtNOS* T-DNA insertion lines, reported to be deficient in production of NO, bacteria grew more rapidly compared to wild type ([Zeidler et al., 2004](#)). These results imply that NO is a key molecule in plant immunity.

1.4.2.1 Roles of S-nitrosylation in plant immunity

Increasing evidence has shown the bioactivity of NO can be transferred through a redox-dependent posttranslational modification, S-nitrosylation, by the addition of an NO moiety to a cysteine (Cys) thiol to form an S-nitrosothiol. S-nitrosylation can regulate protein structure, leading to modulation of protein activity ([Wang et al., 2009b](#), [Yun et al., 2011](#)) or localization ([Mengel et al., 2013](#), [Tada et al., 2008](#)). However, as a small gaseous molecule, NO can cross cell membranes and react with the antioxidant glutathione (GSH) to form S-nitroglutathione (GSNO), which is proposed to function as a major NO reservoir in plants ([Yu et al., 2012](#)). In plants, an enzyme S-nitrosogluthathione reductase (GSNOR) can turnover S-nitrosothiols ([Hematy et al.](#)) and modulate SNO levels. In *Arabidopsis*, GSNOR loss-of-function mutant *gsnor1-3* increase SNO levels and impair plant basal defence ([Feechan et al., 2005](#)). Increasing lines of evidence demonstrated that S-nitrosylation of defence-related proteins regulate plant immunity ([Wang et al., 2006](#), [Yun et al., 2011](#), [Bellin et al., 2013](#)).

NPR1, an important regulator of the SA-mediated signalling pathway, can be S-nitrosylated in response to pathogen attack (Figure 1.5). In the resting cells, NPR1 localizes to the cytoplasm in the form of oligomer by intermolecular disulphide bonds. Upon pathogen infection NPR1 is S-nitrosylated at Cys¹⁵⁶, predictably close to the multimerization interface of NPR1, and this redox modification can enhance disulphide bonds formation, subsequently Polymer NPR1 mainly localize in cytoplasm, inhibiting SA-mediated defence response. NPR1 monomer formation can be enhanced by mutation at Cys¹⁵⁶, suggesting S-nitrosylation of NPR1 at Cys¹⁵⁶ driven by pathogen challenge is a key mechanism in plant defence ([Tada et al., 2008](#)).

It has also been shown that S-nitrosylation of salicylic acid-binding protein 3 (AtSABP3) at Cys²⁸⁰ can inhibit its carbonic anhydrase activity, which is required for defence gene expression in *Arabidopsis* ([Wang et al., 2009b](#)). Recently, another

important protein, the NADPH oxidase RBOHD, which is the major enzyme to catalyse ROS production and mediate cell death development, was found to be S-nitrosylated during the plant defence response. Specifically, the NADPH oxidase activity of RBOHD can be suppressed by NO through S-nitrosylation of Cys⁸⁹⁰ *in vitro* and *in vivo*. Computer modelling suggests that S-nitrosylation of Cys⁸⁹⁰ in RBOHD may prevent the binding of its cofactor flavin adenine dinucleotide (FAD), impairing its enzymatic activity. It was also shown that S-nitrosylation of RBOHD reduced reactive oxygen intermediate production and cell death development in response to pathogen attack. Mutation of Cys⁸⁹⁰ blocked S-nitrosylation and increased ROS accumulation in the immune response, which suggested that pathogen recognition drives S-nitrosylation of RBOHD at Cys⁸⁹⁰ and suppresses its NADPH oxidase activity at later stages of the defence response. This may function as a negative loop to prevent excessive HR formation (Yun et al., 2011). These findings highlighted above suggest that S-nitrosylation of key regulators of plant immunity can control plant disease resistance.

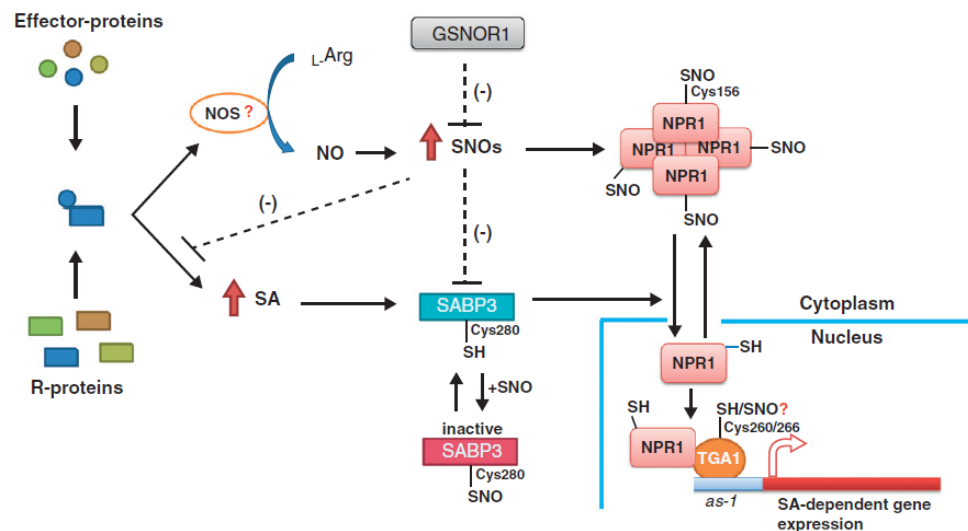


Figure 1.5 S-nitrosylation of proteins in plant defence.

Upon pathogen attack, key regulators of SA-mediated signalling in plant immunity could be S-nitrosylated, resulting in changes of their activity and roles in plant immunity. Adapted from Yu et al (2012) and more detail was described in the reference and text.

1.4.2.2 NO mediates plant development and stress response through S-nitrosylation

In addition to immune responses, it has also been shown that NO is involved in a variety of processes by regulating protein function and signalling based on S-nitrosylation. The observation that the GSNOR loss-of-function mutant, *gsnor1-3*, showed changes in a number of developmental programmes relative to wild-type confirmed the pivotal roles of S-nitrosylation in plants ([Feechan et al., 2005](#), [Yun et al., 2011](#)). Several proteomic experiments were performed to identify the S-nitrosylated proteins during responses to biotic and abiotic stress. About 60 proteins were identified based on mass spectrometry in *Arabidopsis* suspension cultures following treatment by GSNO. Interestingly, most of these proteins are related to stress responses and cellular signalling ([Lindermayr et al., 2006](#)). In a recent study, 62 S-nitrosylated proteins were identified in resting *Arabidopsis* cells and 20 of them were increasingly S-nitrosylated in response to cold stress ([Puyaubert et al., 2014](#)). Similarly, salt stress can enhance the level of a few endogenously S-nitrosylated proteins ([Fares et al., 2011](#)). These data suggest that NO might regulate stress responses by protein S-nitrosylation. Recently, proteomic assays were performed in *gsnor1-3* mutants, which results in elevated SNO levels, and 926 S-nitrosylated proteins were identified, such as APX and TRI1. These data provide a valuable resource to understand the mechanism of NO signalling in plants ([Hu et al., 2015](#)).

NO can possibly directly regulate plant development and stress responses by modulating hormone signalling. The *Arabidopsis* auxin receptor, the F-box protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1), was shown to be S-nitrosylated at Cys¹⁴⁰ and this modification enhanced the interaction between TIR1 and IAA (indole-3-acetic acid). This promoted the degradation of Aux/IAA, an active repressor of auxin signalling, resulting in activation of signal transduction ([Terrile et al., 2012](#)). Moreover, NO was shown to negatively regulate abscisic acid (ABA) signalling by S-nitrosylation of SnRK2.6 (Sucrose nonfermenting 1-Related protein Kinase 2.6) at Cys¹³⁷ in the plant salt stress response ([Wang et al., 2015](#)). These research findings suggest that NO can govern hormone signalling pathways by regulating key regulators via S-nitrosylation.

1.4.2.3 Regulation of S-nitrosylation in plants

GSNOR was firstly identified to govern the context of SNO catabolism in plants

(Feechan et al., 2005, Malik et al., 2011, Chaki and Lindermayr, 2014). In the loss-of-function mutant *gsnor1-3*, GSNOR activity was reduced and consequently the SNO level was significantly increased. In contrast, the level of SNO in the gain-of-function mutant *gsnor1-1* was remarkably reduced (Feechan et al., 2005). The results here indicate that GSNOR is a key regulator of SNO levels in plants. Interestingly, a GSNOR-like enzyme SNO-CoA reductase, encoded by the *alcohol dehydrogenase 6 (ADH6)* in *Arabidopsis*, was identified recently (Anand et al., 2014). Similarly, the total SNO level was increased in *adh6* mutants as in *gsnor1-3* mutants (Feechan et al., 2005, Anand et al., 2014). These findings suggest that GSNOR and SNO-CoA reductase play key roles in the regulation of S-nitrosylation in plants.

Importantly, it is shown that plant Thioredoxin-h5 (TRXh5) has denitrosylation activity and is capable of removing NO directly from S-nitrosylated proteins, which is different from GSNOR or SNO-CoA reductase (Tada et al., 2008, Kneeshaw et al., 2014). The report of denitrosylation activity assays *in vitro* and *in vivo* confirmed that *Arabidopsis* TRXh5 exhibits the ability to remove NO directly (Kneeshaw et al., 2014). The disease susceptible phenotype of the *nox1* mutant, can be restored to wild-type by overexpressing *TRXh5*. Also, the high SNO level in *nox1* mutants was restored to that in wild-type when overexpressing *TRXh5* (Kneeshaw et al., 2014). These results imply that TRXh5 can perform denitrosylation activity to modulate SNO levels in plant immunity.

1.4.2.4 NO in the regulation of gene expression

Transcriptome analysis suggests that NO could reprogramme gene expression (Palmieri et al., 2008, Ahlfors et al., 2009). Although there is no experimental evidence to show that NO can directly regulate gene expression, Previous data suggested that NO may modulate transcript levels of stress-related genes in response to pathogen challenge (Parani et al., 2004) and cold stress (Cantrel et al., 2011) in plants. In this context, NO production was significantly increased in response to chilling treatment within few hours and the cold-response genes such as *CBF1* and *CBF3* (C-repeat binding factors) expression were up-regulated, but their expression were impaired in double mutants *nia1nia2* plant, which is deficient in NO production, in response to low temperature. Moreover, transgenic *Arabidopsis* overexpressing *rat*

neuronal NOS (nNOS) enhanced NO level and mRNA of *CBF1*, which confirmed that NO can regulate *CBF* transcripts in response to stress response ([Shi et al., 2012](#)). Further, NO was reported to modulate transcriptional activity of transcription factors in eukaryotes ([Sha and Marshall, 2012](#)) to modulate transcriptome. The transcriptional activity of human transcription factor myocyte enhancer factor 2 (MEF2) was shown to be inhibited by NO via S-nitrosylation ([Okamoto et al., 2014](#)). Also, regulation of transcriptional activity of transcription factors by S-nitrosylation in *Arabidopsis* was identified. TGA1 ([Lindermayr et al., 2010](#)) and AtMYB30 ([Tavares et al., 2014](#)), transcriptional activity might be regulated by this redox modification *in vitro*, although the mechanisms associated with S-nitrosylation of TGA1 and atMYB30 *in vivo* have not been investigated. Recently, NO was proposed to regulate gene expression by mediating the N-end rule pathway ([Hu et al., 2005](#), [Gibbs et al., 2011](#), [Licausi et al., 2011](#)). In this system, proteins with cysteine at N-terminal can be targeted by proteolysis for degradation in the presence of NO, but these targeted proteins were stable in the presence of NO scavenger c-PITO, suggesting NO could mediate the N-end rule pathway to govern the proteins stability. Moreover, plant specific transcription factors, such as VII type ethylene response factors (ERFs), are targets of the N-end rule pathway and NO could regulate these transcription factors by governing the N-end rule pathway. For example, NO can regulate transcription factor ERFs stability by the N-end rule pathway to control stress-related genes expression in response to stress response ([Gibbs et al., 2014](#)). In animals, cysteine residues of zinc finger domain in transcription factors are proposed to function as NO sensors and it is possible that S-nitrosylation of zinc finger domains regulate their transcriptional activity ([Kröncke, 2001](#)). Collectively, NO can mediate transcription factor stability or activity to regulate gene expression by various mechanism, but the mechanism of NO is associated with transcription is still poorly understood.

1.5 Transcription factors in plant immunity

1.5.1 Transcription factors in plant defence

Plants have developed a variety of strategies to protect themselves from environmental stresses. Transcription factors are a class of key proteins that regulate

gene expression through recognizing and binding to *cis*-elements in the promoter of target genes ([Eulgem and Somssich, 2007](#), [Ciftci-Yilmaz and Mittler, 2008](#)). Transcription factors are involved in various plant processes including development and defence responses ([Singh et al., 2002](#)). Further, transcription factors have been identified to play pivotal roles in plant defence responses ([Singh et al., 2002](#), [Baena-Gonzalez et al., 2007](#), [Ciftci-Yilmaz and Mittler, 2008](#), [Li et al., 2013](#)). In plant hormone pathways, transcription factors act as an important element to switch on/off signalling by controlling stress-related genes expression, such as the WRKY transcription factor WRKY70.

The WRKY transcription factor WRKY70 has been shown to regulate plant defence pathways. Briefly, WRKY70 functions as a positive regulator of the SA pathway in the plant defence response and activates SA-related gene expression, while suppressing jasmonic acid (JA)-related gene expression ([Li et al., 2004](#), [Li et al., 2006](#)). Overall, lots of transcription factors have been revealed for plant stress responses ([Singh et al., 2002](#), [Ciftci-Yilmaz and Mittler, 2008](#), [Chen et al., 2012](#), [Kielbowicz-Matuk, 2012](#), [Mizoi et al., 2012](#)). Based on the development of microarray and next generation Sequencing approaches, it is becoming easier to identify transcription factors and their target genes in response to environmental stimuli. However, the mechanisms that regulate these transcription factors typically still need to be elucidated.

1.5.1 Zinc finger containing transcription factors in plants

Regulation of stress-related gene expression is an important strategy to cope with environmental changes, implying significant roles of transcription factors in plant defence responses. Zinc finger containing transcription factors comprise a large transcription factor family in plants and act as an important regulator of nucleic acid binding and gene expression ([Rizhsky et al., 2004](#), [Ciftci-Yilmaz and Mittler, 2008](#), [Fukushima et al., 2012](#), [Kielbowicz-Matuk, 2012](#)). Zinc finger domain in plant transcription factor is capable of binding DNA as its special structure forming by cysteines and/or histidines coordinate a zinc atom(s). According to the number of cysteine and and histidine, the zinc finger containing proteins were classified into 9 types (C2H2, C8, C6, C3HC4, C2HC, C2HC5, C4, C4HC3 and CCCH)

([Ciftci-Yilmaz and Mittler, 2008](#)). Further, it was demonstrated that the zinc finger domain in these transcription factors is the motif required for DNA binding activity. For example, the C2H2-zinc finger domain was identified to bind to the AG/CT motif in *cis*-acting elements in the promoter region of target genes ([Fukushima et al., 2012](#)). Many studies have suggested that the DNA binding domain, C2H2-zinc finger domain, could directly regulate stress-response gene expression through binding a *cis*-element in the target promoter ([Asako et al., 2000](#), [Englbrecht et al., 2004](#), [Kielbowicz-Matuk, 2012](#), [Shi et al., 2014](#)). Further, transcription factors can function in different stresses, such as salt, drought and light ([Rizhsky et al., 2004](#), [Davletova et al., 2005](#), [Rai et al., 2013](#), [Perveen et al., 2013](#), [Rai et al., 2014](#)). Besides, many zinc finger transcription factors have been characterized in plant stress responses ([Ciftci-Yilmaz and Mittler, 2008](#), [Tian et al., 2010](#), [Hamel et al., 2011](#), [Kielbowicz-Matuk, 2012](#)). However, transcriptome profiling demonstrated that pathogens could induce a number of zinc finger genes, indicating zinc finger proteins may be involved in plant immune responses ([Libault et al., 2007](#), [Asai et al., 2014](#)) and recently meta-analysis was performed and found zinc finger domain may also involved in plant disease resistance ([Dobón et al., 2015](#)). The studies highlighted above demonstrate that zinc finger proteins may function in plant immunity. However, very few zinc finger proteins have been found to function in plant immunity.

1.6 Objectives

It has been well established that NO bioactivity can be transferred by the redox dependent modification, S-nitrosylation ([Yu et al., 2014](#)). Importantly, S-nitrosylation is emerging as a key post-translational modification in plant development and stress responses ([Wang et al., 2006](#), [Yu et al., 2012](#)). As discussed earlier, a number of key regulators of signalling pathways in plants have been shown to be S-nitrosylated in developments and stress responses.

The detailed role of NO in plant immunity has begun to be characterized and some target proteins of NO have been uncovered. Transcriptome analysis suggests that NO could also reprogramme gene expression ([Palmieri et al., 2008](#), [Ahlfors et al., 2009](#)). However, little is known about the mechanism(s) of how NO could control

gene expression. In order to better understand the mechanisms of NO-mediated transcriptional reprogramming, we identified a sub-group of SNO-regulated genes (*SRGs*), transcripts of which can be strongly induced by NO in response to pathogen challenge. It is possible that SRGs are targets of NO in plant immunity, because SRGs belong to C2H2-type transcription factor family, which are proposed as possible targets for NO regulation in animals. Moreover, in animal systems NO has been implicated to modulate C2H2-type zinc finger structure as well as DNA binding activity *in vitro* ([Garbán and Bonavida, 2001](#), [Kröncke, 2001](#), [Sha and Marshall, 2012](#)). However, nothing is known of the potential underlying mechanisms.

It is therefore possible that NO may control SRGs transcriptional activity to reprogramme the plant transcriptome in response to redox cues. This project will investigate this hypothesis, possibly providing important new insights into NO signalling in plants, which might guide future plant breeding or genome engineering strategies.

Chapter

2 Materials and methods

2.1 Plant growth conditions and plant lines

All *Arabidopsis* lines were sterilized with 70% (v/v) ethanol for 1 minute and 2% (v/v) bleach for 8 minutes, finally washed three times with H₂O, and then were placed on the 1/2 MS and keep in 4 °C for two days. After being cold stratified, the plants were put in culture room with 12 hours light. About two weeks, the plants were transferred to soil in the short day (8 hours light) or long day growth room (16 hours light).

Table 2.1 *Arabidopsis* lines

name	ID	Description	origin
WT	Col-0	<i>A. thaliana</i> ecotype Columbia	Loake Lab stock
<i>atgsnor1-3</i>	<i>At5g43940</i>	T-DNA insertion resulting in the inactivation of <i>atgsnor1</i> gene	GABI-Kats
<i>35S::GSNOR</i>	<i>35S::GSNOR</i>	Overexpression of <i>GSNOR</i> in Col-0	Loake Lab stock
<i>srg1</i>	<i>At3g46080</i>	T-DNA insertion, SALK_119663	SALK
<i>srg2</i>	<i>At3g46090</i>	T-DNA insertion, GABI_404D05	GABI
<i>srg3</i>	<i>At5g59820</i>	T-DNA insertion, SAIL_1213_C07	SALK

2.2 Genomic DNA extraction and genotyping

For genotyping, genomic DNA from *Arabidopsis* leaves was extracted. About 50 mg young leaves were collected and ground with tissue lyser (Qiagen) for 1 minute at 25 shakes per second. Subsequently, 400 µL of extraction CTAB buffer [200 mM Tris-Cl (pH7.4), 250 mM NaCl, 25 mM EDTA, and 1% SDS] was added to resuspend thoroughly and then spin down at 13000 g for 3 minutes. 300 µL supernatant was transferred to a new 1.5 ml tube and 300 µL 2-propanol was added to precipitate DNA 30 minutes. After that the samples were centrifuged 5 minutes to pellet DNA and washed twice with 70% (v/v) ethanol, finally the DNA was dissolved with 50 µL H₂O and 1 µL was used for genotyping by PCR.

Table 2.3 Primers used for genotyping of T-DNA insertion mutants.

Mutants	Primers
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<i>srg1</i>	RP: ATGTCTCCTCATGTGACCACC, LP: CAGATTAATCGAGTTGCTGCC LB: TACGAATAAGAGCGTCCATTTT
<i>srg2</i>	RP: TGGACAAGGATCCAAGAAGTG, LP: TTCAATCGGTGATCATATGGAGG LB: GCTTCCTATTATATCTTCCCAAATTACCAATACA
<i>srg3</i>	RP: TTGTTTTTATTCGTGATGGGG, LP: TCTCTTAAGCTACGCGGTGTC LB: ATATTGAACATCATACTCAT
<i>gsnor1-3</i>	RP: CAGCAGCCTCATGACCTAGAATACAAGGAA LP: GGATCGATAAGGTTCCCAGTCTAGCTAGGTA LB: ATAATAACGCTGCGGACATCTACATTTT

2.3 RNA extraction and qRT-PCR

2.3.1 RNA extraction

RNA extraction was performed according to the RNA extraction kit instruction (Agilent Plant RNA Isolation Mini Kit). Briefly, 100 mg plant leaves were smashed with mortar and pestle in liquid nitrogen and homogenate with 500 μ L extraction buffer. Then transfer the buffer to prefiltration column and spin down for 3 minutes at 16000 g, and then add 500 μ L 2-propanol to flue through. Centrifuge the mix through mini isolation column and wash twice with 500 μ L wash buffer at 16000 g for 2 minutes. Finally, RNA was eluted with 20 μ L Rnase-free H₂O.

2.3.2 RT-PCR

cDNA was synthesized from 2 μ g RNA using oligo (dT) primers and reverse transcriptase according to the instruction (First-Strand cDNA Synthesis Kit, invitrogen).

The qRT-PCR was performed according to The LightCycler® 480 Real-Time PCR System (Roche). Briefly, cDNA synthesis were amplified and quantified by LightCycler® DNA Master SYBR Green I mix and LightCycler system. The relative gene expression value was determined using *UBQ10* as the reference. All the experiments were performed in triplicate.

Table 2.4 List of primers for RT-PCR

Gene	Forward primers	Reverse primers
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<i>PR1</i>	CGTTCACATAATTCCCACGAG	TCAGTGAGACTCGGATGTGC
<i>UBQ10</i>	AGATCCAGGACAAGGAAGGTATTC	CGCAGGACCAAGTGAAGAGTAG
<i>ACTIN 1</i>	GTGCTCGACTCTGGAGATGGTGTG	CGGCGATTCCA GGGAACATTGTGG
<i>SRG1</i>	TCATCGTGCAAGCCACAAGA	GGAGTGAGAAAGCCTCACCA
<i>SRG2</i>	GCTGCGGGGGAGATGAAC	TCGGACAAGGATGAGACGTT
<i>SRG3</i>	TGTCCCATATGTGGAGTGGA	ATTGTCCACCATCCCTAGACT

2.4 Generation of construction

2.4.1 Cloning and transformation in *E. coli*

All the genes examined for this project were amplified with Phusion High-Fidelity DNA Polymerase from Col-0 cDNA (prepared in 2.3.2) by PCR. The PCR product was separated on agarose gel and purified using Gel Extraction Kit (Qiagen). For Gateway® Cloning (Invitrogen), 2 μ L purified fragments were mixed with 2 μ L pDONR221 and kept at 25 °C for 1 hour for BP reaction with 1 μ L BP Clonase (Invitrogen). For classic ligation, the PCR product was digested with appropriate restriction enzyme and then cloned into the pGEM-T easy vectors with T4 ligase (Promega). The resulting product was transformed into *E.coli* DH5 α competent cells by heat shock method. Briefly, resulting ligation were added to 40 μ L competent cell DH5 α and then the mixture were kept on ice for further 20 minutes and then heated at 42 °C for 42 seconds. The mixture were kept on ice for another 2 minutes and incubated at 37 °C shaker for 1 hours after adding 1 ml LB broth. The transformed competent cell were spreaded on the LB agar with appropriate antibiotic and kept at 37 °C for 16-18 hours.

2.4.2 Generation of construction for protein expression

For SRG recombinant protein expression, the *SRG* genes were amplified with appropriate restriction site and then cloned into MBP-tagged expression vector pMAL-c5X. The entry vectors of *Topless*, *MAPK3* and *MAPK6* in pDONR221 generated in 2.4.1 and destination vector pDEST-HisMBP or pDEST15 were used for LR reaction.

Table 2.5 List of primers for protein expression *in vitro*

protein	Primers	Vectors	Cloning sites
MBP-SRG1	F: CATGCCATGGATGGTTGCGAGAAGTGAGGA R: CGGAATTC AGAAATCGTTCTTCCCAACTC	pMAL-c5X	<i>Nco</i> I / <i>Bam</i> HI
MBP-SRG2	F: CGGGATCCATGGTTGCGATATCGGAGATCAAG R: CGGAATTCCTTAAGTCAAGAAATCGTTCTTCCC	pMAL-c5X	<i>Nco</i> I / <i>Bam</i> HI
MBP-SRG3	F: CGGGATCCATGGTTGCGATATCGGAGATCAAG R: CGGAATTCATAAACTGTTCTTCCAAGCTCCA	pMAL-c5X	<i>Bam</i> HI / <i>Eco</i> R I
GST-MAPK3	F: GGGGACAAGT TTGTACAAAAAAGCAGGCTTCAT GGACGGTGGTTCAGGT R: GGGGACCACTTTGTACAAGAAAGCTGGGTTCTA TTGCTGATATTCTGG	pDONR221/ pDEST15	attB1/attB2
GST-MAPK6	F: GGGGACAAGT TTGTACAAAAAAGCAGGCTTC ATGGACGGTGGTTCAGGT R: GGGGACCACTTTGTACAAGAAAGCTGGGTT CTATTGCTGATATTCTGG	pDONR221/ pDEST15	attB1/attB2
GST-Topless	F: GGGGACAAGT TTGTACAAAAAAGCAGGCTTC ATGTCTTCTCTTAGTAGAGAGCTC R: GGGGACCACTTTGTACAAGAAAGCTGGGTT AAGAGGTGTTGGAACAGGTGAC	pDONR221/ pDEST15	attB1/attB2

2.4.3 Generation of constructions for plant transformation and for transient transcriptional repressor assay

In order to generate transgenic plant lines, the *SRGs* in the pDONR221 were cloned into different destination vectors by LR reaction with LR Clonase enzyme (Invitrogen), according to protocol. Specifically, destination binary vector pGWB11 for C terminal FLAG tag (*35S* promoter), pEarlyGate 103 for C-terminal GFP tag (*35S* promoter) and the pGWB3 for C-terminal GUS reporter gene (no promoter).

For transient transcriptional repression assay, the *SRG* genes were fused to GAL4-DB. Specifically, the vectors were described previously ([Guo et al., 2013](#)) and were digested with restriction enzymes (*Eco*R I and *Cla* I). Also, the *SRG* gene was amplified with restriction enzyme sites. The subsequent sequences were ligated to the vector and transformed to *E.coli*.

2.4.4 Generation of construction for yeast two hybrid and BiFC

For yeast two hybrid (Y2H) and bimolecular fluorescence complementation (BiFC), the sequences in pDONR221 were used to do LR reaction with pDEST32 and pDEST22 to generate AD fusion and BD fusion Y2H vectors or with p1112 and p1113 to generate C-terminal or N-terminal YFP ([Bos et al., 2010](#)).

Table 2.6 List of primers for plant transformation

Gene	Primers	Vectors and tags	Cloning site
<i>SRG1</i>	F: <u>GGGGACAAGT TTGTACAAAAAAGCAGGCTTC</u> ATGGTTGCGA GAAGTGAGGAA R: <u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> AGAAATCGTTCTTCCCAACTC	pEarleyGate103 / pGWB11	attB1/attB2
<i>SRG2</i>	F: <u>GGGGACAAGT TTGTACAAAAAAGCAGGCTTC</u> ATGGTTGCGA GAAGTGAGGA AA R: <u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> ACTCCAAGAAATCGTTCTTCC	pGWB11/ pEarleyGate103	attB1/attB2
<i>SRG3</i>	F: <u>GGGGACAAGT TTGTACAAAAAAGCAGGCTTC</u> ATGGTTGCGA TATCGGAGAT R: <u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> ATAAACTGTTCTTCACCGC	pGWB11 / pEarleyGate103	attB1/attB2
<i>Pro^{srg1}</i>	F: <u>GGGGACAAGT TTGTACAAAAAAGCAGGCTTC</u> CGCAATTCAGCATTTTCAAGTT R: <u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> TTTGAATCAACTTAGTGACTTC	pGWB3	attB1/attB2
<i>Pro^{srg2}</i>	F: <u>GGGGACAAGT TTGTACAAAAAAGCAGGCTTC</u> TCAGGGTAAGAATAAGATGCTTG R: <u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> TTTCCTTTCTATGATCGGTTTTTT	pGWB3	attB1/attB2
<i>Pro^{srg3}</i>	F: <u>GGGGACAAGT TTGTACAAAAAAGCAGGCTTC</u> TATCGCAACCATTTTTCTTCTGATGA R: <u>GGGGACCACTTTGTACAAGAAAGCTGGGTT</u> AGAAAAATGGTTGCGATATCGGAGA	pGWB3	attB1/attB2
<i>SRG1</i>	F: <u>GCTCTAGAATGGTTGCGAGAAGTGAGGAA</u> R: <u>GGACTAGTtcaagaaatcgttcttccaact</u>	GAL-DB	<i>Xho</i> I / <i>spe</i> I
<i>SRG2</i>	F: <u>CCGCTCGAGATGGTTGCGAGAAGTGAGGAA</u> R: <u>GGACTAGTttaactccaagaaatcgttcttcc</u>	GAL-DB	<i>Xho</i> I / <i>spe</i> I
<i>SRG3</i>	F: <u>CCGCTCGAGATGGTTGCGATATCGGAGATC</u> R: <u>GGACTAGTtcaataaactgttcttccaagc</u>	GAL-DB	<i>Xho</i> I / <i>spe</i> I

2.5 Recombinant protein expression and purification

For recombinant protein expression, constructs of pDEST-HisMBP and pDEST15 with gene of interest were transformed into *E. coli* strain B121 (DE3). Growing the singal colony overnight at 37 °C and adding 1 mL of overnight culture to 100 mL LB medium to grow until OD₆₀₀=0.4, then 0.1 mM IPTG were added and growth for additional 4 hours at 22 °C. Cells were collected, washed in pre-cooled PBS buffer and resuspended in pre-cooled PBS buffer. For MBP-tagged protein purification, Amylose Magnetic Bead was used and performed according to instruction (*NEB*). GST-tagged protein purification was performed using Glutathione Sepharose 4B bead and carried out according to its protocol (GE healthcare life sciences).

2.6 Biotin switch assay *in vitro*

Firstly, recombinant protein was desalted by spin down through Micro Bio-Spin P6 columns (BioRad). The desalted protein was incubated in HEN buffer (100 uL, 250 mM Hepes-NaOH pH 7.7, 1 mM EDTA and 0.1 mM neocuproine) with or without NO donor at 25 °C for 20 minutes in dark. After that, NO donor was removed using Micro Bio-Spin P6 columns and then add 300 µL blocking buffer (2.5% SDS and 20 mM NEM in HEN buffer) to block the free thiol groups in 50 °C for 20 minutes. The extra MMTS was removed by acetone precipitation. Finally, labelling buffer (HEN buffer with 1 mM biotin-HPDP and 5 mM sodium ascorbate) were used to dissolve protein for 1 hour at room temperature and then load sample for western blot with anti-biotin antibody.

2.7 Biotin switch assay *in vivo*

Plant total protein was extracted with 200 µl extraction buffer [1X HEN buffer, 1 time protease inhibitors (Roche), 0.5% Triton-X100] and then kept in 50 °C for 20 minutes after adding 400 µL blocking buffer. Then precipitated the protein with 1.2 mL acetone in -20 °C. The precipitated protein was labelled by 250 µL labelling buffer (1 mM biotin-HPDP and 5 mM sodium ascorbate) and 20 µL were collected as the input. The biotin labelled proteins were pulled with Streptavidin overnight in 4 °C

and the resulted proteins were subjected to western blot.

2.8 Protein interactions

2.8.1 GST pull down

For GST pull down assay, MBP-tagged and GST-tagged protein were used. About 100 μ g of purified recombinant protein or empty tag were incubated together in PBS buffer and kept in 4 °C for 2 hours with Amylose Magnetic Bead and washed 6 times with PBS then the bound proteins were used for western blot with anti-GST antibody. On the contrary, Glutathione Sepharose 4B bead was used to pull MBP or MBP-tagged proteins and western blot was analysed against anti-MBP antibody.

2.8.2 Yeast two hybrid

GAL4-based yeast two hybrid (Y2H) system was performed for testing proteins interaction according to Bos et al., (2010). Briefly, the BD and AD vector were co-transformed into yeast strain AH109, which contains the *HIS3* (Growing on lack histidine medium) and *LacZ* report genes (Converting X-Gal to produce blue), and the successful transformant colony were selected on the synthetic dextrose lacking leucine/tryptophan (SD-L-T) medium and then subsequently the colonies were grown on synthetic dextrose lacking leucine/tryptophan/histidine (SD-T-L-H) with 3-Amino-1,2,4-triazole (3-AT) to avoid the false interaction, as 3-AT could inhibit basal yeast *HIS* gene expression. Additionally, the correct colonies were also grown on SD-T-L-H with X-alpha-gal to test the expression of *lacZ*. If the protein interacts with each other, the corresponding colony could grow on SD-T-L-H with 3-AT and also induced *lacZ* expression.

2.9 Degradation assay

For the in vitro degradation assay of MBP, MBP-SRG1 and MBP-SRG3, 100 μ g of recombinant protein was added to 500 μ g of plant total proteins prepared from 4-week-old Col-0 and keep at 30 °C for indicated times. 15 μ L of samples were loaded on SDS-PAGE gel for western blot against anti-MBP antibody.

2.10 EMSA

Purified recombinant proteins and ordered probes were used for EMSA and performed as described previously ([Fukushima et al., 2012](#)). Briefly, the oligo probes are annealed using PCR machine with the program (95 °C 10 mins, 60 °C 30 mins, 18°C 30 °C). 100 ng of annealed probes are labbled with γ -³²ATP by T4 polynucleotid kinase (Femantas, UK) in 37 °C for 1 hour and then free γ -³²ATP was removed with sephadex G50 column (GE healthcare). Before adding labbled probe, recombinant protein in binding buffer (20 mM HEPES PH7.9, 1 mM DTT, 50 mM KCl, 5% Glycerol, 5 mM MgCl₂, 50 µg/ µL BSA) were kept at 4 °C for 20 mins and then 1µL probe was added for binding reaction at RT for 20 mins. Resulting reaction mix were subjected to 6% denature polyacrylamide gel and the autoradiation signal was exposed after the gel is dried.

2.11 Plant transformation

2.11.1 Plant stable transformation

The correct plastid was transformed into *Agrobacterium* strains GV3101 by freeze/thaw shock method ([Sakhno et al., 2002](#)) and then the plasmid was transformed into *Arabidopsis* by *Agrobacterium tumefaciens*-mediated transformation with floral dipping method ([Zhang et al., 2006](#)). Briefly, the *Agrobacterium* with desired recombinant plasmid was grown in 5 mL LB broth with appropriate antibiotic for overnight at 37 °C shaker for 2 days and the third day 2 mL culture were diluted to 250 mL for further 16-20 hours (OD=1.5 to 2). The *Agrobacterium* cells were collected and resuspended in 5% (w/v) sucrose, and 0.02% (v/v) silwet L-77 were added and mixed just before use. The health *Arabidopsis* lines with inflorescences, flower and siliques were used. The inflorescences were dipped in the sucrose solution with *Agrobacterium* cells for 30 seconds followed by keeping in dark for 24 hours, and then moved to growth room for seeds production. After collecting seeds, place them on the 1/2 MS with appropriate antibiotic for selection and the survived plant were transformed to soil for growing. Genotyping PCR was done to confirm the transformant from genomic DNA. The homozygous transformants with single insertion were selected for further experiment based on the segregation of 3:1.

2.11.2 Plant transient transformation

2.11.2.1 Transient transformation in tobacco leaf

The *Agrobacteria* GV3101 carrying indicated binary vector was grown for overnight at 28°C and pellet 1 mL of culture. Using infiltration Buffer (50 mM MES pH 5.6, 2 mM Na₃PO₄ and 100 µM Acetosyringone) to resuspend the pellet and adjust the OD=0.1 or 0.5. Infiltrate buffer by syringe (no needle) against the lower (abaxial) epidermis of a tobacco leaf and mark the areas. For localization, the photographs were taken after 72 hours post transformation under Leica SP5 (Germany) confocal microscopy. The excitation laser wavelength is 488 nm for GFP and the UV is for DAPI.

2.11.2.2 Transient transformation in *Arabidopsis* protoplasts

Leaves were collected from about 4 to 5 weeks *Arabidopsis* under short day conditions. The upper epidermal surface was stuck to autoclave tap and use magic tap to remove the lower epidermal surface gently. The autoclave tap with leaves was put in a Petri dish containing 10 ml of enzyme solution [20 mM MES, pH 5.7, 400 mM Mannitol, 20 mM KCl, 10 mM CaCl₂, 0.1% (w/v) BSA, 1% (w/v) Cellulase and 0.25% (w/v) Macerozyme] and shake for about 1 hour until the buffer become green which means the protoplasts were dissolved in enzyme buffer. The protoplasts were collected by centrifugation for 3 minutes at 100 g and use pre-cooled W5 buffer (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM CaCl₂, 5 mM MKCl and 5 mM Glucose) to remove the enzyme solution. After this then keep the protoplast on ice for 1/2 hour and pellet the protoplast and resuspend protoplast with MMG buffer (4 mM MES pH 5.7, 0.4 M Mannitol and 15 mM MgCl₂).

100 µL of protoplasts were added to the tube containing 10 µL of DNA (4 µg of transcription factor, 5 µg of promoter with Luciferase and internal control) and mix thoroughly. Then 110 µl of PEG/Ca solution (40% PEG, 0.2 M Mannitol and 0.1 M CaCl₂) were added and keep for 10 minutes at room temperature (about 23-25 °C). 0.44 ml of W5 solution was added to the reaction and then pellet the protoplasts and resuspend in 1.5 ml of W5 solution and keep under light for 18 hours. The protoplasts were collected by spin down at 100 g for 2 minutes and for further experiment.

2.12 Transcriptional repression activity assay

The effector plasmid, reporter plasmid and internal plasmid were together used for transient transcriptional repressor assay using *Arabidopsis* protoplast and performed as described elsewhere ([Guo et al., 2013](#)). After transformation, protoplasts are kept at RT under light for 12-16 hours and then collected for luciferase activity assay using Dual-Luciferase Report Assay System (Promega, USA) with Multimode Plate Reader M5 (Molecular Device, USA).

2.13 Pathogenesis assay

2.13.1 Pathogen culture

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) with or without avirulence gene were used. Bacterial cells were spreaded on LB agar medium. The signal colony was grown in LB liquid medium overnight with rifampicin.

2.13.2 Pathogen infection assay

For seedlings, about 10-day-old plant from 1/2 MS plate were treated with *Pst* DC3000 or *Pst* DC3000 carrying *avrRpm1* were adding to 1/2 MS liquid medium (OD=0.5) were harvest in the indicated time and for ROS or RNA assay. For adult plant leaves of 4 to 5 weeks plants grown in short day condition were inoculated by syringe injection with *Pst* DC3000 or *Pst* DC3000 carrying *avrRpm1* and the samples were taken in the indicated time and for further experiments.

2.13.3 Bacterial growth assays

The 4 to 5-week plantwere infected by syringe injection with *Pst* DC3000 or *Pst* DC3000 carrying *avrRpm1* (OD=0.0002) for 3 days and then 10 pieces of 0.2 cm² infected leaves were collected and put in 1.5 mL tube with two steel ball, following ground using tissue lyser (Qiagen). H₂O (500 µL) were added to resuspend the lysis tissue with vortex and dilute the lysis tissue with 1:10 until to 10⁻⁶ (*Pst* DC3000/*avrRpm1*) or 10⁻⁷ (*Pst* DC3000). Flowing dilution, 20 µL of samples were spread on LB medium with rifampicin and keep at 30°C for 2 days. Finally, the

amounts of colony growing were calculated according to the dilution.

2.14 Histological assay

2.14.1 Gus staining

In order to analysis of Gus expression or Gus activity, the *Arabidopsis* seedlings or tissues with or without treatment indicated were submerged in Gus staining buffer (8 mg X-Gluc in 10 ml of 0.16 M NaPO₄ pH 7.0 with 0.4 M K₃[Fe(CN)₆] and K₄[Fe(Lickwar et al.)₆] and 0.4 M EDTA) for 4 hours at 37 °C in dark. After distaining the tissue with 70% ethanol until remove the background.

2.14.2 DAB and NBT staining

To explore the ROS burst in the indicated plant line or treated lines, the *Arabidopsis* seedlings or tissue with or without treatment were submerged in the NBT staining buffer (0.5 mg/mL NBT in PBS buffer) for 3 hours or DAB staining solution (1 mg/mL) for 8 hours at room temperature in dark and destain the tissue with 70% (v/v) ethanol until the green colour was removed completely.

2.14.3 Trypan blue staining

To determine the progress of infection or cell death, *Arabidopsis* leaves with or without treated were stained in trypan blue solution (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL water and 10 mg of trypan blue) by boiled for 2 min. After cooling to room temperature, the samples were destained by 2.5g/mL chloral hydrate until the samples are clean. The samples were rinsed with water and viewed under an Olympus (Japan) microscope

2.15 SDS-PAGE and western blot

Protein samples with SDS-loading buffer (0.5% bromophenol blue, 0.1 M DTT, 10% glycerol, 2% SDS and 0.05 M Tris-Cl pH 6.8) were heated at 90 °C for 5 minutes and then loaded on the 12% SDS-PAGE at 100 V for 2 hours. The gel was stained by instant blue or transferred onto PVDF membrane by wet transfer in the transfer buffer (25 mM Tris-HCl pH 7.6, 192 mM glycine and 20% methanol) at 90 V

for 1 hour. The membrane were blocked in PBST buffer (PBS with 0.1% Tween 20) with 5% non-fat milk for 30 minutes at room temperature with agitation and then washed with PBST once. Then membrane was washed twice with PBST after incubating with primary antibody for 1 hour. After that, membrane was incubated with secondary antibody for 1 hour at room temperature with agitation. Subsequently, the blot was washed 3 times with PBST and incubated with 1 mL LumiGLO (0.5 mL solution A and 0.5 mL solution B) (Thermo) for 1 minute at room temperature. Finally, the blot was exposed to film for varies time and develop the film in the X-ray developer.

Table 2.7 List of antibodies

primary antibody	Host species	Manufacturer
anti-MBP HRP-linked	Mouse	New England Biolabs
anti-GST HRP-linked	Goat	Cambridge BioScience
anti-biotin HRP-linked	goat	Cell Signaling Technology
anti-FLAG	mouse	Sigma
Secondary antibody	Host species	Manufacturer
anti-mouse IgG HRP-linked	goat	Cell Signaling Technology

Chapter

3 Identification of SNO-regulated genes (SRGs) in plant immunity

3.1 Introduction

Plants have developed a sophisticated immune system against potential pathogen infection. Following pathogen recognition, second messengers, including ROS ([Yoshioka et al., 2009](#)), NO ([Leitner et al., 2009](#)), Ca^{2+} ([Du et al., 2009](#)) and MAPKs ([Meng and Zhang, 2013](#)) function in early events during subsequent immune signalling. As a key redox signalling molecule, NO plays pivotal roles in plant immunity ([Yu et al., 2012](#)). Treatment with NO donor has been proposed to enhance *Arabidopsis* resistance to bacterial pathogen, meanwhile application of an NO scavenger, cPTIO, could reduce resistance. These data implied application of NO positively mediated plant immunity ([Delledonne et al., 1998](#)). In addition, pathogen recognition also elevated NO levels ([Chandok et al., 2003](#)). Whereas, in the *Arabidopsis* T-DNA insertion line *AtNOS*, which is claimed to be deficient in production of NO, bacteria grew to a higher titre ([Zeidler et al., 2004](#)). Collectively, these results suggest that NO is involved in plant immunity.

It has also been proposed that NO could regulate gene expression during attempted pathogen infection ([Delledonne et al., 1998](#), [Bellin et al., 2013](#)). Furthermore, it has been shown that NO could directly modify protein activity during plant immunity ([Dong, 2004](#), [Wang et al., 2009b](#), [Yun et al., 2011](#)). For example, S-nitrosylation, a post-translational modification mediated by adding a NO moiety to a highly reactive protein Cys thiol group to form an S-nitrosothiol ([Hematy et al.](#)), could alter the protein activity ([Bellin et al., 2013](#), [Yu et al., 2012](#)). In *Arabidopsis* SNO levels are thought to be controlled by GSNOR, and the GSNOR loss of function mutant, *gsnor1-3*, exhibits susceptibility to the pathogen infection, which suggests SNO levels are important for plant immunity ([Feechan et al., 2005](#)). Furthermore, SNO was shown to affect SA signalling during plant defence response by S-nitrosylation ([Wang et al., 2009b](#), [Yun et al., 2011](#)). In this context, S-nitrosylation

of SA-binding protein 3 (SABP3) at Cys²⁸⁰ inhibited its SA binding activity and compromised disease resistance against *Pst*DC3000 /*avrRps4* (Wang et al., 2009b). In addition to SABP3, some other proteins have been identified that can be S-nitrosylated upon pathogen challenge, such as NPR1 (Zeidler et al., 2004) and the NADPH oxidase, RBOHD (Yun et al., 2011). Microarray analysis also showed NO could regulate gene expression, indicating NO may also regulate plant defence by reprogramming transcripts (Parani et al., 2004).

Although the roles of NO in plant immunity have begun to be characterized and some target proteins of NO have been identified in response to attempted pathogen infection, it is still not clear how NO signals are translated into global changes in gene expression. In this chapter, we identified prototypic, redox regulated transcription factors that are transcriptionally regulated by NO during plant immunity.

3.2 Identification of SNO-regulated genes, *SRGs*, in plant defence

Changes in redox status are a key feature of immunity response to attempted pathogen infection (Yu et al., 2012). In order to fully understand the roles of NO in plant immunity, we aimed to identify and characterise transcription factors that are responsive to both NO and pathogen challenge. Analysis of both public data bases (Parani et al., 2004) and our in-house microarray data (Yun et al., unpublished) for genes up-regulated by NO, we identified a sub-group of C2H2 Zinc Finger containing transcription factors that were rapidly activated by NO, including *SRG1* (at3g46080), *SRG2* (at3g46090, also known as *ZAT7*) (Ciftci-Yilmaz et al., 2007) and *SRG3* (at5g59820, also known as *ZAT12*) (Asako et al., 2000, Rizhsky et al., 2004, Davletova et al., 2005). We found that the transcripts of *SRGs* can be strongly induced by NO and the transcript levels increased 56.4, 11 and 3541 times, respectively. These data implies *SRGs* could be regulated by NO at the transcriptional level. In addition, based on the previous published data, these *SRGs* could also be up-regulated by pathogens or elicitors (Ramonell et al., 2005, Libault et al., 2007, Hiruma et al., 2011), indicating *SRGs* may be involved in plant immunity.

To determine if these *SRGs* are NO regulated in response to pathogen infection, reverse-transcription (RT)-PCR was carried out and the wild type Col-0 and *gsnor1-3*

lines were used. The *PR1* expression was used as a pathogen-related marker gene and its expression, as expected (Feechan et al., 2005), was delayed in the *gsnor1-3* mutants, which is GSNOR deficient and has high GSNO level (Figure 3.1). RT-PCR results showed these *SRGs* were all induced by *Pst* DC3000 carrying avirulence gene *avrB*. Interestingly, the expression patterns of *SRGs* upon pathogen in the *atgsnor1-3* mutant were different, compared with Col-0. Therefore, these *SRGs* are transcriptionally regulated by NO or SNO.

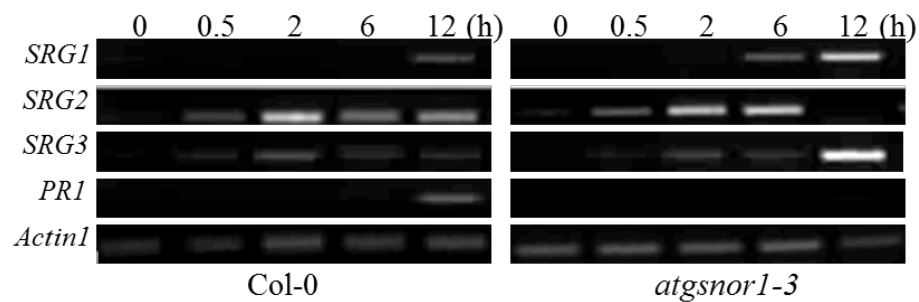


Figure 3.1 RT-PCR analysis of *SRG* expression in response to attempted pathogen infection.

SRG transcripts were analyzed in Col-0 and *atgsnor1-3* lines upon the challenging by *Pst* DC3000/*avrB* ($OD_{600}=0.2$) at the indicated times post inoculation. Pathogen-regulated gene *PR1* was used as a marker for the activation of plant immune expression. *Actin1* was used as an internal control (22 cycles).

3.3 Gene sequence analysis

To date, *SRG2* and *SRG3* have only been reported to be implicated in abiotic plant stress responses, but not in plant immunity. Importantly, the sequence alignment of *SRGs* showed a high level of DNA sequence conservation between these genes. In order to understand the evolutionary relationship between them and other zinc finger TFs, a neighbour joining phylogenetic tree of *SRGs* in *Arabidopsis* was generated based on the C2H2 zinc finger genes from *Arabidopsis* (Englbrecht et al., 2004). This analysis revealed that *SRGs* were classified into a small group (Figure 3.2). Although there is another gene *at3g46070* that is also closely related to *SRGs*, public transcriptomic data suggests this gene cannot be induced by NO. Collectively, these

data suggest that *SRG* genes might have related functions in *Arabidopsis*.

Furthermore, public transcriptomics data implies *SRGs* are induced during the immune response. *SRG1* expression was increased to 3.4 fold in the enhanced disease resistance 1 (*edr1*) mutant, but only increased to 2.11 fold compared to Col-0 in the *edr1* phytoalexin deficient 4 (*pad4*) double mutant (Ramonell et al., 2005), suggesting *SRG1* may function as a positive regulator in plant immunity. In addition, global gene expression profiling data has also shown *SRG* expression was induced in response to an oomycete downy mildew pathogen (Asai et al., 2014) and the fungus PAMP chitin (Libault et al., 2007). These data implies that *SRGs* might be involved in plant immunity.

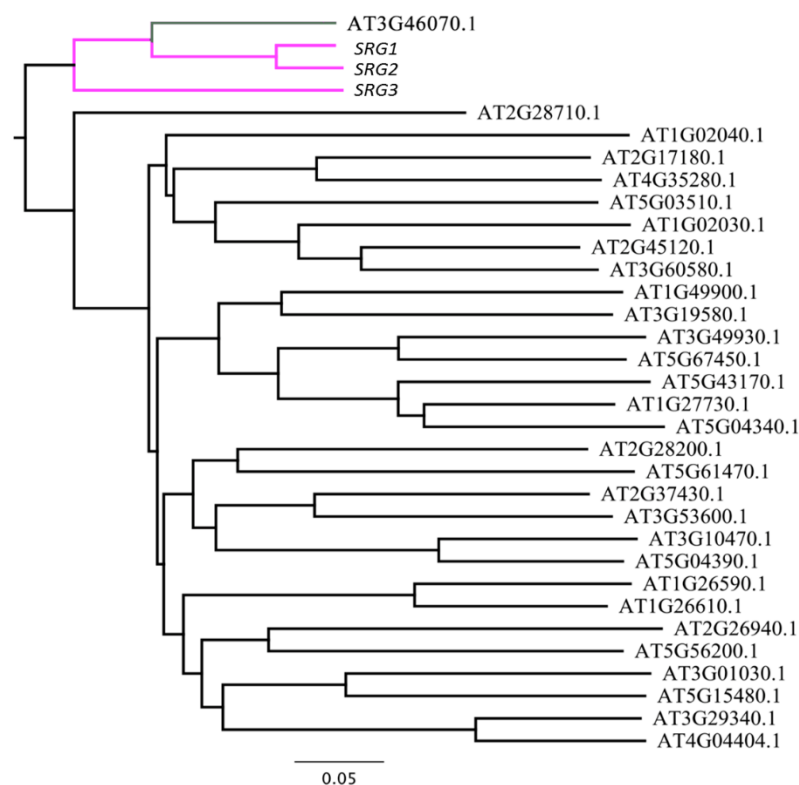


Figure 3.2 Phylogenetic tree of C2H2 type transcription factor in *Arabidopsis*.

Sequences of C2H2 type transcription factor in *Arabidopsis* obtained from GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). The phylogenetic tree was clustered using the neighbor joining method based on the sequence by the software Geneious version 6.0.5.

It is possible that *SRGs* are important regulators of (S)NO accumulation during the pathogen-triggered nitrosative burst and subsequently they might mediate cell response by modulating gene expression.

3.4 Generation of transgenic plant lines *SRG1::GUS* and *SRG3::GUS*

As mentioned above, *SRGs* are responsive to both NO and pathogens. To gain further insights into the *SRGs* expression pattern, the promoter region of two *SRG* genes were amplified from *Arabidopsis* Col-0 genomic DNA and fused with the reporter gene β -glucuronidase (*gus*) and transgenic plants expressing the resulting transgene were generated. The *SRG1* promoter (1894 bp) and *SRG3* promoter (2345 bp) were amplified as described (Figure 3.3). We tried but failed to amplify the promoter of *SRG2*. The transgenic plants harbouring the *SRG1::GUS* or *SRG3::GUS* were obtained and the plants were screened on 1/2 MS plates with kanamycin to obtain homozygous lines with a single insertion. To confirm transformants, primers for the *GUS* gene were used and genotyping was performed. The predicted product of 560 bp was obtained from the tested transgenic plants, whereas no product was obtained from wild type Col-0 genomic DNA (Figure 3.4). The T3 generations were used to confirm these experiments.

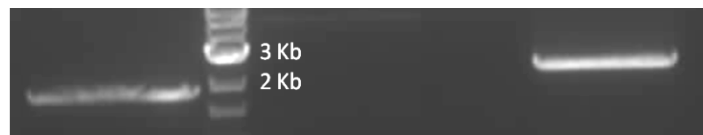


Figure 3.3 Amplification of the promoter region of *SRGs*.

The promoter region of *SRG1* and *SRG3* were amplified from Col-0 genomic DNA by High proof Phusion polymerase. The *SRG1* promoter (left) and, the *SRG2* promoter (middle) and the *SRG3* (right) are shown. M (DNA marker), DNA size of 2 kb and 3 kb are indicated in the figure.

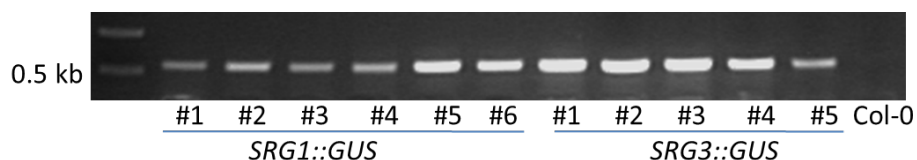


Figure 3.4 Genotyping of transgenic plants with GUS specific primers.

The genomic DNA of the independent transgenic plant lines with *SRG1::GUS* or *SRG3::GUS* were amplified by *GUS* specific primers designed to give a product ~0.5 kb. Col-0 genomic DNA was used as negative control. 0.5 kb of DNA fragment was highlighted in the figure.

3.5 *SRG::GUS* are induced by NO

Public data and our RT-PCR data (Figure 3.1) demonstrated *SRGs* might be regulated by NO in the plant defence response. In order to confirm our hypothesis that they could be induced by NO, we assayed *SRG* expression in transgenic plants with both *GUS* reporter genes and qRT-PCR following treatment with the NO donor, sodium nitroprusside (SNP).

Firstly we treated the transgenic plants harboring *SRG1::GUS* and found SNP could induce GUS activity at 6 h and GUS activity was even stronger at 24 h, indicating *SRG1* was inducible by NO donor, SNP (Figure 3.5). We also tested whether application of the NO inhibitor cPTIO (2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), an NO scavenger, could block SNP-induced *SRG1* expression. When treated with cPTIO, SNP-induced *GUS* expression of *SRG1::GUS* lines was reduced, suggesting that the NO scavenger cPTIO could blunt NO-mediated *SRG1* expression.

We also tested the *SRG3* expression pattern in response to NO in *SRG3::GUS* lines using SNP. GUS activity was induced strongly at 1 h, which suggested that *SRG3* expression was activated more rapidly than *SRG1* by SNP (Figure 3.6). Moreover, the application of cPTIO reduced GUS activity in response to SNP, indicating cPTIO also blocked SNP-induced *SRG3* expression.

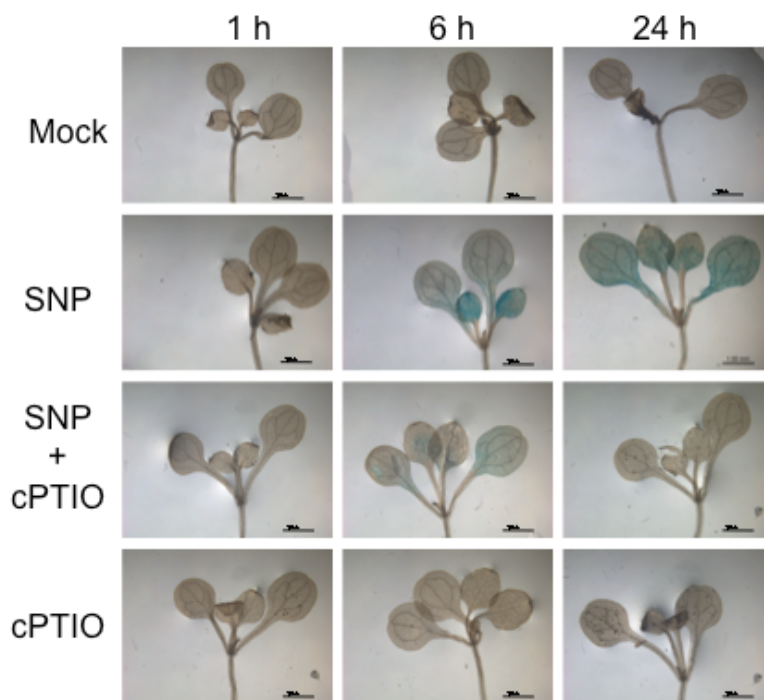


Figure 3.5 Accumulation of GUS activity upon SNP treatment of *SRG1::GUS* line.

GUS activity in the *SRG1::GUS* line in response to SNP (300 μ M) and SNP plus cPTIO (200 μ M) was analyzed by GUS staining.

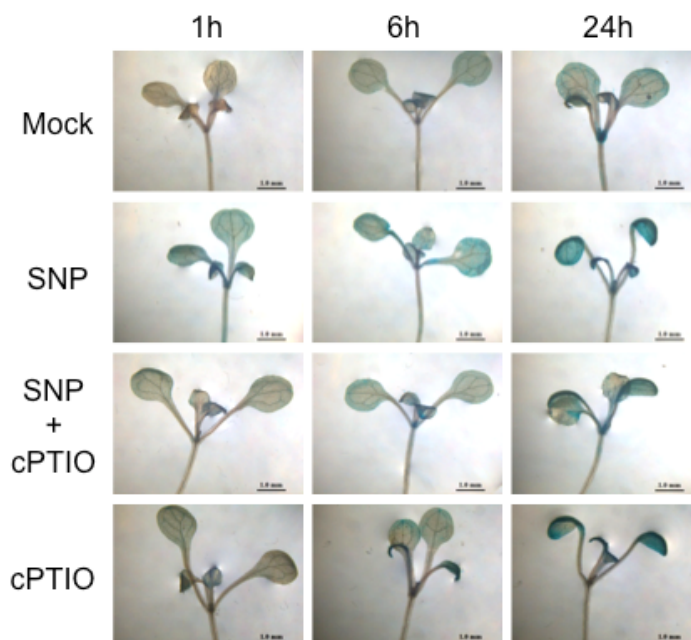


Figure 3.6 Accumulation of GUS activity upon SNP treatment of *SRG3::GUS* lines.

GUS activity in a representative *SRG3::GUS* line in response to SNP (300 μ M) and SNP plus

cPTIO (200 μ M) were analyzed by GUS staining.

qRT-PCR was also carried out to confirm *SRG1* expression data in response to NO donor. Ten-day-old Col-0 seedlings treated with SNP or SNP plus cPTIO and then samples were collected at 6 h and 24 h and subjected to qRT-PCR. This data showed SNO induced transcripts of *SRG1* at 6 h and 24 h post SNP treatment (Figure 3.7). As expected, application of cPTIO blunted *SRG1* expression in response to SNP. The qRT-PCR data also suggested that the NO scavenger cPTIO could block SNP induction of *SRG1* expression.

We also examined *SRG2* expression in response to NO using qRT-PCR. The results showed that SNP activated *SRG2* transcription at 6 h and the expression increased 8-fold compared with the mock at 24 h. Surprisingly, application of cPTIO did not alter *SRG2* expression, which implies that cPTIO could not block SNP-induced *SRG2* expression.

We also used qRT-PCR to quantify *SRG3* expression in response to NO. Interestingly, the *SRG3* expression increased at 6 h, but then decreased at 24 h. However, application of NO scavenger cPTIO blunted the *SRG3* expression induced by SNP. The results showed *SRG3* is NO inducible and it was reversible by cPTIO.

Collectively, the data showed that *SRG* genes are NO inducible and cPTIO could block this induction, except in the case of *SRG2*.

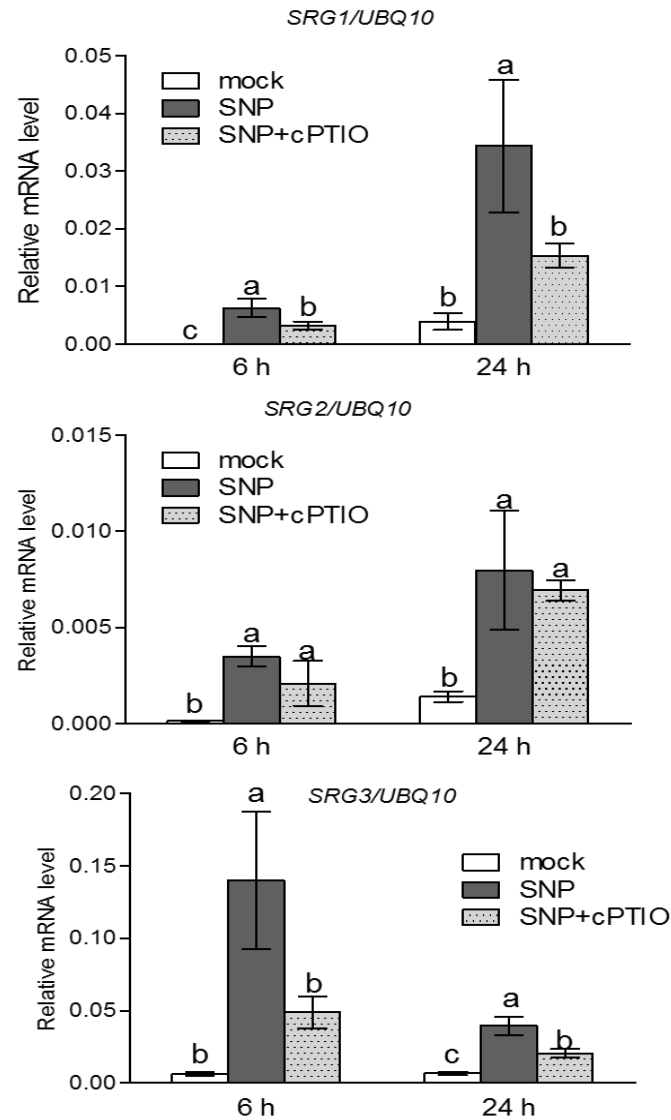


Figure 3.7 Transcript levels of *SRG* genes were determined following treatment with the NO donor, SNP.

Ten-day-old Col-0 seedlings were treated with SNP (300 μ M) with or without cPTIO (200 μ M) and samples at the indicated times post treatment were collected. mRNA level were then assayed by qRT-PCR. *UBQ10* was used as internal control. Error bars represent the SD from at least 3 independent biological replicates. Different letters indicate the significant difference between mock determined by student *t* test with $P < 0.05$.

3.6 *SRGs* are inducible in response to attempted pathogen infection

As *SRG* genes may be involved in the plant defence response, we tested *SRGs*

transcript levels in response to attempted pathogen infection. We inoculated wild type Col-0 leaves with *Pst* DC3000 carrying the avirulence gene *avrRpm1* and collected samples at the indicated times, followed by qRT-PCR. Figure 3.8 showed that all *SRG* genes were up-regulated in the presence of *Pst* DC3000/*avrRpm1*. However, there was a slight difference amongst their expression patterns. Transcription of *SRG1* was more sensitive to this pathogen and retained high levels in response to *Pst* DC3000/*avrRpm1*. On the contrary, both of *SRG2* and *SRG3* were induced at 3 hours post inoculation (hpi) and then declined to approximately wild type levels. These data suggested *SRG* genes can be induced by *Pst* DC3000/*avrRpm1* and may be involved in plant immunity.

To verify pathogen-induced *SRG1* and *SRG3* expression, we treated the transgenic *SRG1::GUS* (Figure 3.9a) and *SRG3::GUS* (Figure 3.9b) plants with *Pst* DC3000 or *Pst* DC3000/*avrRpm1*. GUS activity was induced in response to *Pst* DC3000 and *Pst* DC3000/*avrRpm1*, which showed that both *SRG1* and *SRG3* were induced during plant immune function. We also treated these transgenic plants with SA (Figure 3.9), an important immune activator. SA was also found to induce *SRG1* and *SRG3* expression, suggesting *SRG1* and *SRG3* indeed play roles in plant immunity.

Based on the results of qRT-PCR and the response of transgenic plants with *SRG::GUS* reporter genes, our data implies that *SRG* genes are pathogen inducible and may function in plant immunity.

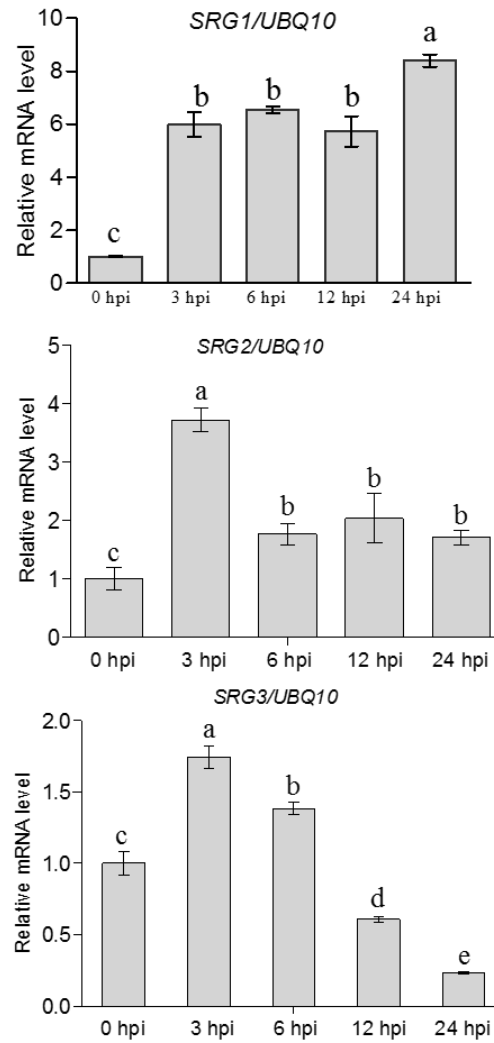


Figure 3.8 *SRG* genes are induced by *Pst* DC3000/*avrRpm1*.

qRT-PCR was carried out to quantify *SRG* gene expression in response to *Pst* DC3000/*avrRpm1* infection. *UBQ10* was used as an internal control. Error bars represent the SD from at least 3 independent biological replicates. Different letters indicate the significant difference between 0 hour post inoculation (hpi) determined by student *t* test with $P < 0.05$.

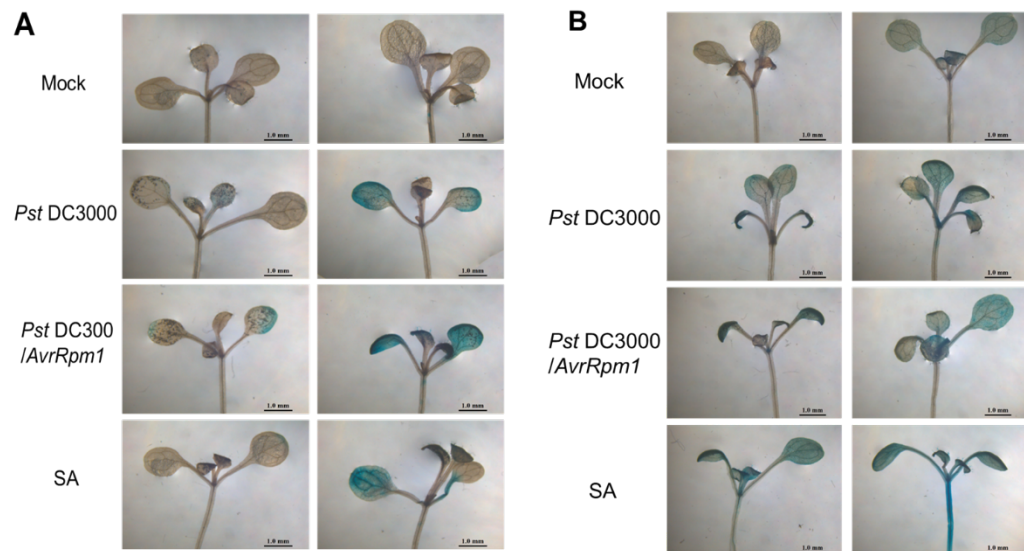


Figure 3.9 Accumulation of GUS activity upon pathogen and SA treatment.

Ten-day-old transgenic plants harbouring *SRG1::GUS* (A) or *SRG3::GUS* (B) at 24 hours post treatment of *Pst* DC3000, *Pst* DC3000/*avrRpm1* or SA were assayed for GUS activity.

3.7 SRG genes encode nuclear proteins

To examine the subcellular localization of SRG proteins, the corresponding coding regions were amplified from *Arabidopsis* cDNA then translationally fused with the green fluorescent protein (GFP), followed by *Agrobacterium tumefaciens* GV3101 mediated transient expression in tobacco leaves. As expected, our data suggested these proteins located to the nucleus, but free GFP was detected throughout the whole cell (Figure 3.10). In order to confirm nuclear localization, we stained tobacco leaves with DAPI (4', 6-diamidino-2-phenylindole), a fluorescent nuclear and chromosome counterstain and monitored SRG1 localization under confocal microscopy (Figure 3.11). SRG1::GFP proteins co-localized with the nucleus revealed by DAPI staining. Additionally, nuclear localization sequence (NLS) fused GFP (NLS-GFP) ([Koyama et al., 2013](#)) was used as a control. These data suggest SRG proteins are nuclear proteins.

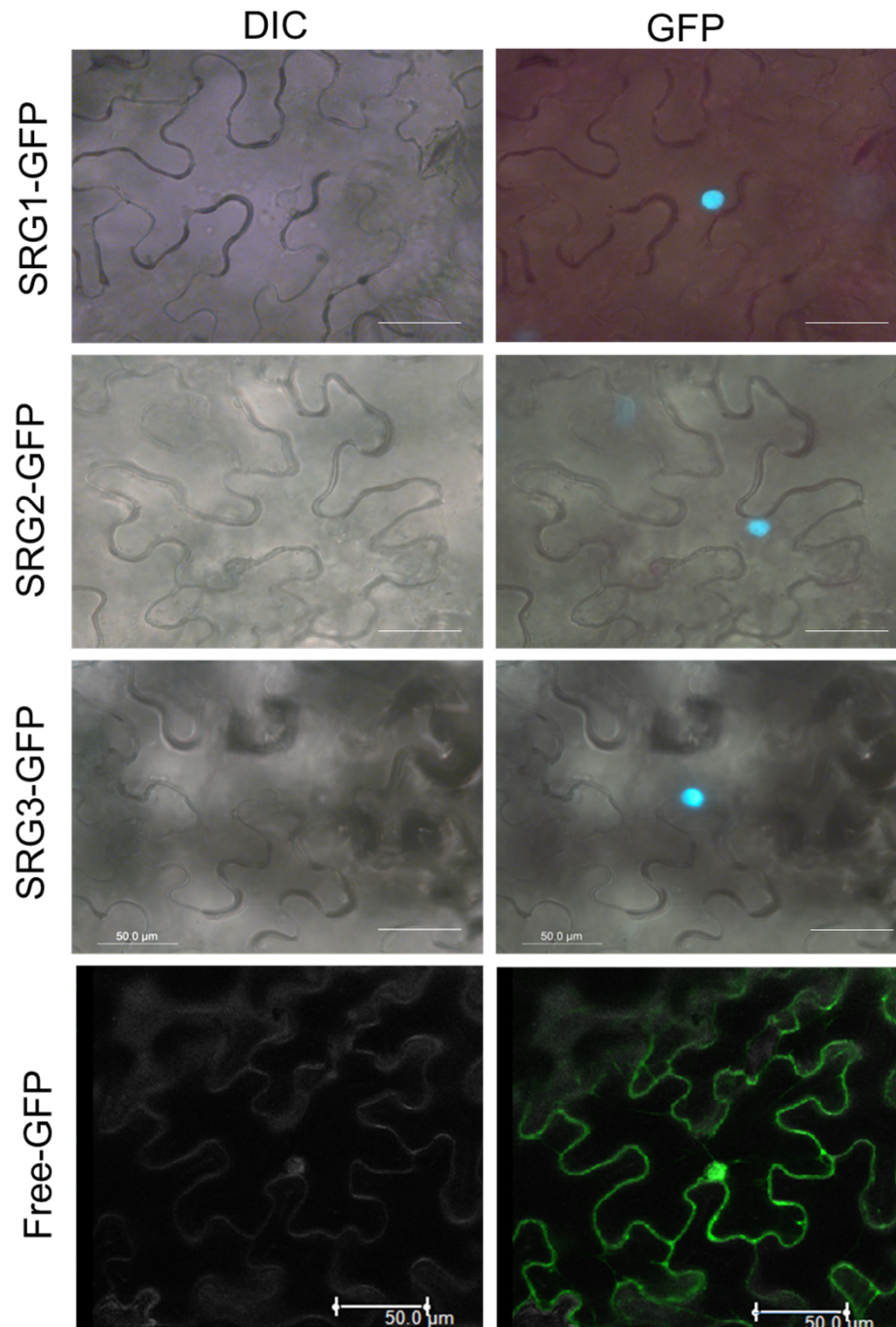


Figure 3.10 Subcellular localization of SRG proteins in tobacco leaves.

SRG1-GFP (upper row), *SRG2-GFP* (middle row), *SRG3-GFP* (lower row) and *GFP* (under lower row) were transiently expressed in tobacco leaves mediated by *Agrobacteria* GV3101, respectively. The photographs were taken at 72 hpi using fluorescence microscopy. Scale bar, 50 µm.

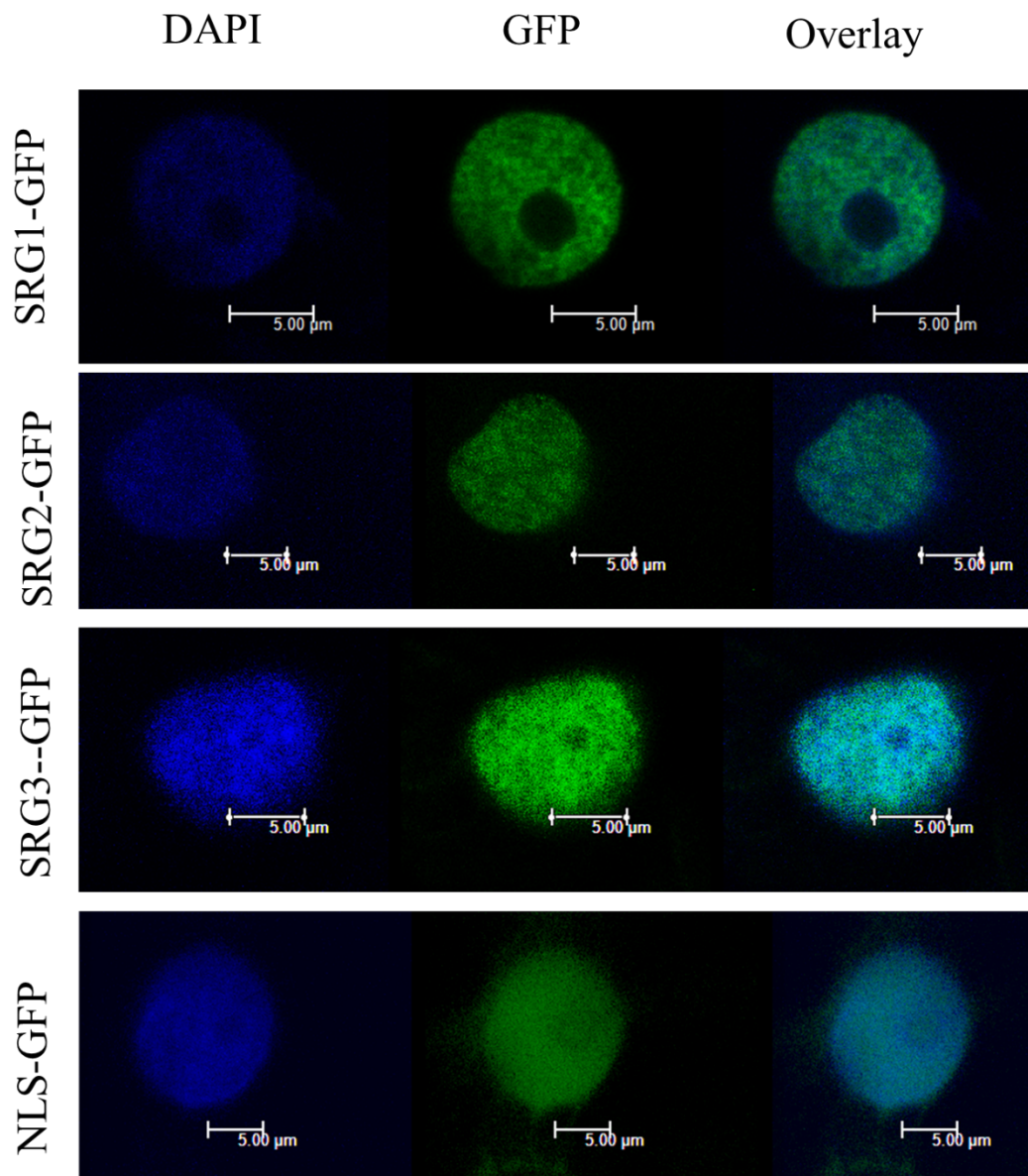


Figure 3.11 SRG proteins localize in the nucleus of tobacco leaves.

The plasmids of *SRG1-GFP* (upper), *SRG2-GFP* (middle) and *SRG3-GFP* (lower) were transformed into tobacco leaves by *Agrobacteria* GV3101 mediated transient expression, respectively. The NLS-GFP and free GFP were used as controls. Photographs were taken 36 hpi under confocal microscopy.

3.8 Discussion

NO has been emerged as the key regulator in plant defence ([Bellin et al., 2013](#), [Yu et al., 2014](#)). As a key redox molecular, NO could change the redox status, which

could regulate redox-sensitive proteins. Although some NO-sensitivity regulators in plant immunity were identified, the global sensitive transcription factors of NO are required to dissect the roles of NO in plant immunity. In this chapter, we identified a small group of NO-response transcription factors, *SRGs*, which might provide the insight of NO signalling in plant immunity.

SRGs were identified by their NO-induced expression from public database ([Parani et al., 2004](#)). We confirmed the results with application of NO donor, SNP, and it induced *SRG* genes expression and the NO scavenger cPTIO blunted this induction. Structurally, *SRGs* belong to C2H2 Zinc Finger transcription factor superfamily. Moreover, the cysteine thiols in zinc finger proteins are recognized as the molecular targets of NO and zinc finger proteins have emerged as prototypic transcription factors for redox regulation ([Kroncke et al., 1994](#), [KRÖNCKE and CARLBERG, 2000](#), [Kröncke, 2001](#)). In addition, NO could regulate downstream genes expression by impairing the DNA binding activity in zinc finger containing transcription factors through S-nitrosylation of cysteine thiols in zinc finger domain, such as Sp1 ([Wang et al., 1999](#)) and Yin-Yang 1 ([Garbán and Bonavida, 2001](#)). Recently, the *Arabidopsis* transcription factor AtMYB30 was reported to be S-nitrosylated that resulted in blocking its DNA binding activity *in vitro* ([Tavares et al., 2014](#)). The increasing evidence implied that NO modulates gene expression by influencing the cysteine thiols via S-nitrosylation and therefore it is possible that *SRGs* are targets of NO and NO could regulate the downstream gene expression by controlling DNA binding activity of *SRGs* through S-nitrosylation ([Sha and Marshall, 2012](#)).

It is also reported that *SRGs* are involved in stress response, such as drought, salt and cold ([Asako et al., 2000](#), [Rizhsky et al., 2004](#), [Davletova et al., 2005](#), [Vogel et al., 2005](#), [Ciftci-Yilmaz et al., 2007](#)) and overexpression of *SRG3* enhanced stress resistance ([Perveen et al., 2013](#), [Rai et al., 2014](#)). The data suggested *SRGs* might have potential roles for improving agricultural production during stress. Here we characterized that *SRGs* may also function in plant immunity as *SRGs* were up-regulated by the bacterial pathogen *Pst* DC3000 both by promoter analysis and qRT-PCR analysis. Interestingly, the expression patterns of *SRGs* were altered in

atgsnor1-3, which exhibited high SNO level. These results suggested (S)NO regulated *SRGs* in plant immunity.

A homologous of *SRGs* in poplar, *PtiZFPI*, was identified to be induced by SA, one of the most important phytohormones involved in plant defence ([Pieterse et al., 2012](#)). Consistently, *SRG1* and *SRG3* were also induced by SA, which providing further evidence that *SRGs* are involved in the defence response. Interestingly, *SRG3* transcripts increased upon *Pst* DC3000/*avrB* inoculation in *sid2* and *gsnor1-3* lines (Unpublished data), relative to wild type Col-0. Both of *sid2* and *gsnor1-3* lines compromised the SA level and SA-mediated defence response and the transcripts of *SRG3* continuously increased in the presence of bacterial infection, which suggested the induction of *SRG3* in response to pathogen maybe independent on SA level. But *SRG3* could be induced by SA and may involved in plant defence, the relationship between *SRG3* and SA is complex and maybe other mechanisms exist to control *SRG3* in plant immunity. However, the exact roles of *SRGs* in immunity and NO signalling need to be examined.

Chapter

4 Molecular studies of SRGs

4.1 Introduction

Nitric oxide (NO) is an active redox molecule and S-nitrosylation of glutathione (GSH) forms GSNO, which can serve as an NO reservoir in plants ([Yu et al., 2012](#), [Bellin et al., 2013](#)). NO modulates protein functions thorough S-nitrosylation, a redox based posttranslational modification ([Nakamura and Lipton, 2011](#), [Hess and Stamler, 2012](#), [Yu et al., 2014](#)). S-nitrosylation regulates protein activity by attachment of an NO moiety to a cysteine thiol to form an S-nitrosothiol ([Bellin et al., 2013](#)). Increasing evidence shows that S-nitrosylation plays pivotal role in plant stress responses, including immunity ([Feechan et al., 2005](#), [Tada et al., 2008](#), [Leitner et al., 2009](#), [Wang et al., 2009b](#), [Yu et al., 2012](#), [de Pinto et al., 2013](#), [Frunghillo et al., 2014](#)). In plants, SNO levels could be regulated by the enzyme GSNOR, which can turnover GSNO, indirectly controlling the levels of S-nitrosylated proteins ([Feechan et al., 2005](#), [Leterrier et al., 2011](#), [Frunghillo et al., 2014](#)). Furthermore, some important proteins involved in plant defence that can be S-nitrosylated have been identified and characterised ([Yu et al., 2012](#), [Bellin et al., 2013](#), [Yu et al., 2014](#)), providing evidence for the role of S-nitrosylation in plant immunity. However, the detailed mechanisms of NO function in plant defence by this redox-based modification, especially how NO signalling is translated upon pathogen infection to reprogramme gene expression remains to be established.

In order to uncover the activity of NO in the control of gene expression in plant immunity, we identified genes up-regulated in response to pathogen challenge or NO treatment, termed SNO-regulated genes (*SRGs*), which may function as important regulator of NO signalling to regulate gene expression in plant defence. SRGs are cysteine-rich zinc finger proteins and zinc finger transcription factors have emerged as prototypic transcription factors for redox regulation in animals ([Wang et al., 1999](#), [KRÖNCKE and CARLBERG, 2000](#), [Kröncke, 2001](#), [Henard et al., 2014](#)). In addition, recent research has shown that NO can affect the DNA binding activity of TFs,

suggesting NO could modulate downstream signalling by altering TF activity; examples include atMYB30 ([Tavares et al., 2014](#)) and AtbZIP16 ([Shaikhali et al., 2012](#)). SRGs have been predicted to contain several functional domains, such as an Ethylene-responsive element binding factor-associated Amphiphilic Repression ([Riechmann et al.](#)) motif ([McGrath et al., 2005](#), [Kazan, 2006](#), [Kagale and Rozwadowski, 2011a](#)), implicated in transcriptional repression. Thus, SRGs could function as suppressors of gene expression.

In this chapter, we undertake molecular studies of SRGs, to uncover how SRGs are regulated by NO.

4.2 Sequence analysis of SRGs

Protein sequence alignment of SRGs showed that they are closely related and have some conserved functional domains (Figure 4.1), which indicates that SRGs may have similar functions. Sequence analysis revealed that there are two conserved zinc finger domains, which has a 2 cysteines and 2 histidines, among these SRGs and they belong to the C2H2-type zinc finger family and contain a QALGGH motif within the zinc finger domain, which constitutes the DNA binding domain ([Englbrecht et al., 2004](#)). Apart from the zinc finger domain, there is also an EAR motif, LxLxL, which might act as a transcriptional repressor ([Kagale and Rozwadowski, 2011b](#)). In addition, SRGs also contain a putative mitogen-activated protein kinase (MAPK) docking site at the C-terminus, which could be recognized and phosphorylated by specific MAPKs ([Meng and Zhang, 2013](#), [Pitzschke, 2015](#)). Transcription factors are regulated not only at the transcriptional level, but also at the post-translational level, by mechanisms such as phosphorylation ([Xing et al., 2002](#)) and ubiquitination ([Devoto et al., 2003](#)). Additionally, SRGs are cysteine-rich proteins and they may function as important regulators and subsequently translate this signal into specific outputs during responses to pathogens. Taken together, these proteins contain several functional domains and potentially play important roles.

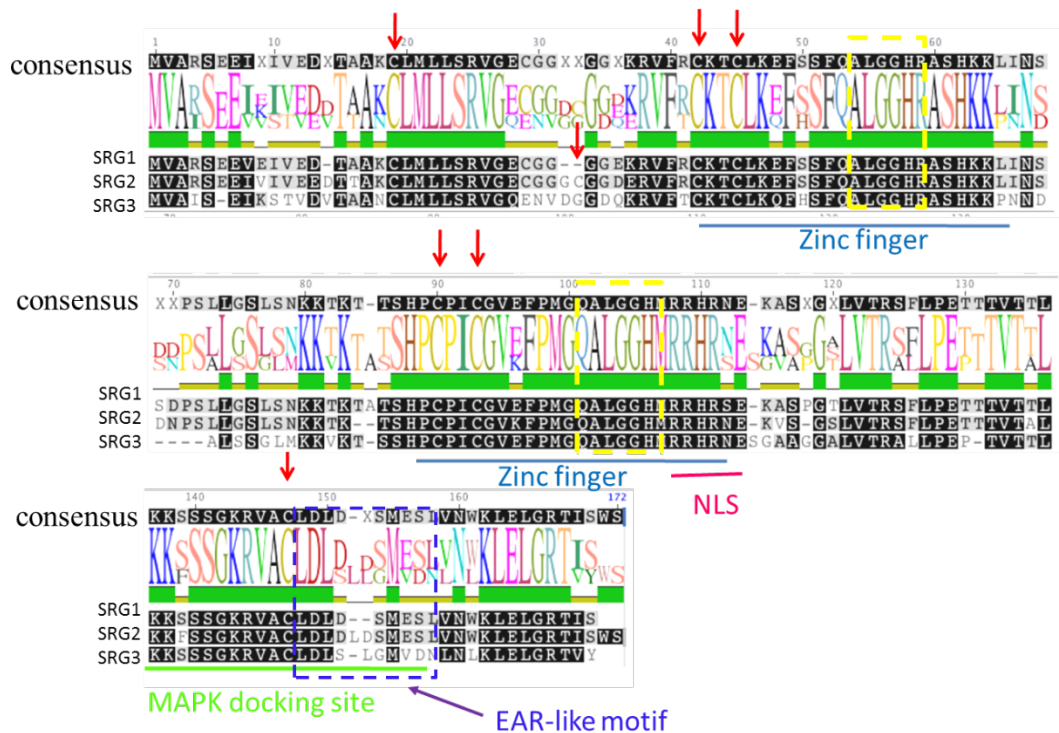


Figure 4.1 Multiple alignment analysis of functional domains in SRGs based on protein primary sequences.

The amino acid sequence analysis of the SRG1, SRG2 and SRG3 were analysed with the software Geneious 6.0.5 packages. Identical amino acids in SRGs are marked in black and grey. The conserved cysteines among SRGs were highlighted by red arrows. The putative functional domains were highlighted in the sequences by different underlines or dotted boxes. The numbers above the sequences showed the amino acid positions. NLS, nuclear localization sequence.

4.3 Expression of recombinant SRGs in *E. coli*

From the protein primary sequence analysis, we found that SRGs contain several important domains, so recombinant proteins were expressed to examine their potential roles *in vitro*. To overexpress the SRGs in *E. coli*, the coding sequences (CDS) of SRGs were amplified from the *Arabidopsis* Col-0 cDNA by PCR with the appropriate restriction sites, respectively. The predicted sizes of 495 bp (SRG1) (Figure 4.2-A), 507 bp (SRG2) (Figure 4.2-B) and 489 bp (SRG3) (Figure 4.3-C) were obtained and cloned into the expression vector pMAL-c5X to generate the recombinant plasmid. The fragment of maltose-binding protein (MBP) tag is present at the N-terminus of

insertion fragment to generate a fusion protein. The sequenced recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) cells for recombinant proteins expression.

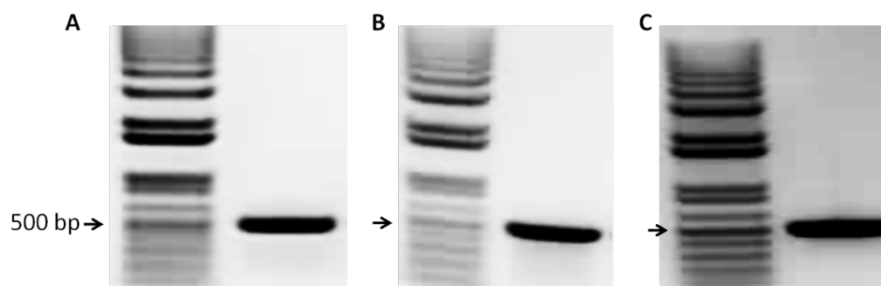


Figure 4.2 Amplification of the CDS of SRGs from *Arabidopsis* Col-0 cDNA.

The full length of coding sequences (CDS) of *SRG1* (A), *SRG2* (B) and *SRG3* (C) were amplified by PCR with restriction sites from Col-0 cDNA. The DNA marker was loaded in the left lane of each gel and DNA of size 500 bp was highlighted by black arrow.

Firstly, different conditions (such as the temperature, IPTG concentration and induction time) for recombinant protein overexpression were performed (Figure 4.3). After confirming the conditions for recombinant protein overexpression, we performed protein purification from *E. coli* using MBP magnetic bead and Figure 4.4 shows the purification of MBP-SRGs, the predicted sizes were all around 60 kDa. In order to confirm whether recombinant protein sequence is correct, MBP-SRG1 was digested in gel using trypsin and the resulting protein was subjected to LC-mass spectrometry. Mass spectrometry showed ~80% coverage of SRG1 (data was not shown), which indicated the recombinant protein was correct.

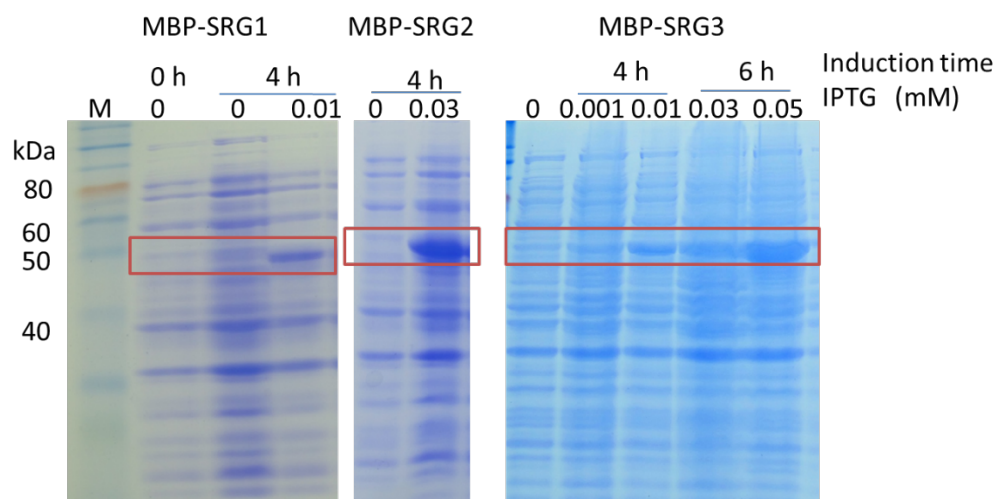


Figure 4.3 Overexpression of MBP-SRGs in *E. coli* BL21 (DE3).

Total proteins were collected before or after isopropyl β -D-1-thiogalactopyranoside (IPTG) treatment and suspended in the lysis buffer and separated on the SDS-PAGE gel for recombinant protein analysis. The SDS-PAGE gel was stained by coomassie blue for 20 minutes. The inducing time and concentration of IPTG were indicated in the figures. The protein marker (M) was labeled in the figure. The predicted sizes of target proteins were highlighted by red box.

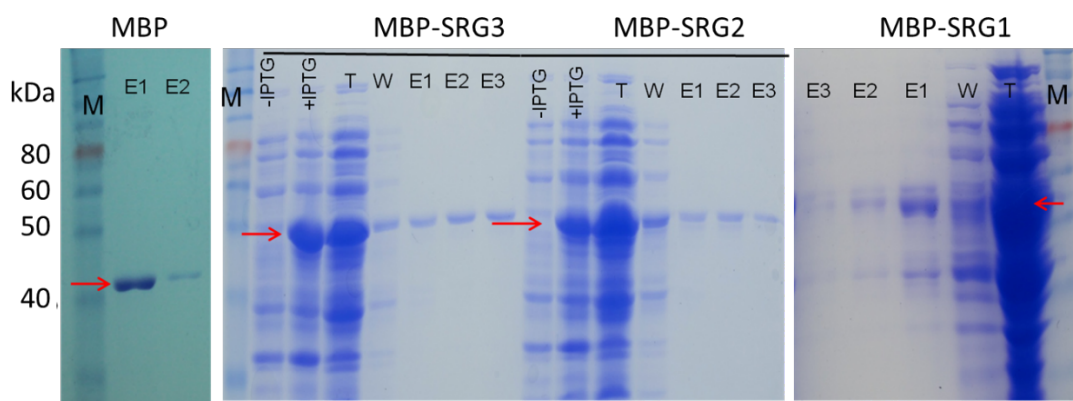


Figure 4.4 Purification of recombinant SRG protein from *E. coli*.

The MBP or recombinant proteins were expressed in *E. coli* and purified with Amylose Magnetic Bead. The recombinant proteins were induced with IPTG (+IPTG) or without (-IPTG) and purified after treatment of IPTG. The red arrows indicate the target protein MBP (left gel), MBP-SRG3 (second gel), MBP-SRG2 (third gel) and MBP-SRG1 (right gel). “M” indicated protein marker and the sizes were presented. “-IPTG” indicates treatment without IPTG and “+IPTG” indicates application of IPTG. “T” presented total soluble protein. “W” presented wash fraction. “E1” presented the first elution fraction. “E2” presented the second elution and “E3”

presented the third elution.

4.4 S-nitrosylation of SRGs *in vitro*

NO could modify and by extension regulate SRG activity by S-nitrosylation of a given cysteine thiol. Thus, we investigated if NO could modify SRGs by S-nitrosylation. The biotin switch technology was developed for analysis of protein S-nitrosylation ([Jaffrey and Snyder, 2001](#)). Therefore, we conducted the biotin switch assay for exploring whether SRGs could be S-nitrosylated. Purified recombinant proteins of MBP-SRGs were incubated with the NO donor, Cys-NO or GSNO in dark for 20 minutes and then subjected to a biotin switch assay to test SNO formation. Figure 4.5 shows that MBP-SRG1 and MBP-SRG3 were S-nitrosylated *in vitro*, whereas MBP-SRG2 was not S-nitrosylated under the conditions tested. In order to test whether S-nitrosylation of SRG1 and SRG3 are dependent on NO donor concentration, different concentrations of GSNO were employed and figure 4.6 shows the extent of S-nitrosylation is enhanced following increasing levels of GSNO. These results suggested that S-nitrosylation of MBP-SRG1 and MBP-SRG3 were GSNO concentration-dependent. Moreover, in the presence of the reducing agent DTT, the S-nitrosylation of SRG1 or SRG3 was significantly reduced, indicating S-nitrosylation of SRG1 and SRG3 were DTT reversible, consistent with a thiol modification of SRG proteins.

Collectively, SRG1 and SRG3 could be S-nitrosylated by NO and these modifications are NO concentration dependent and reversible by the reducing reagent DTT. Furthermore, SRG1 and SRG3 may serve as the targets of S-nitrosylation, a redox-based modification.

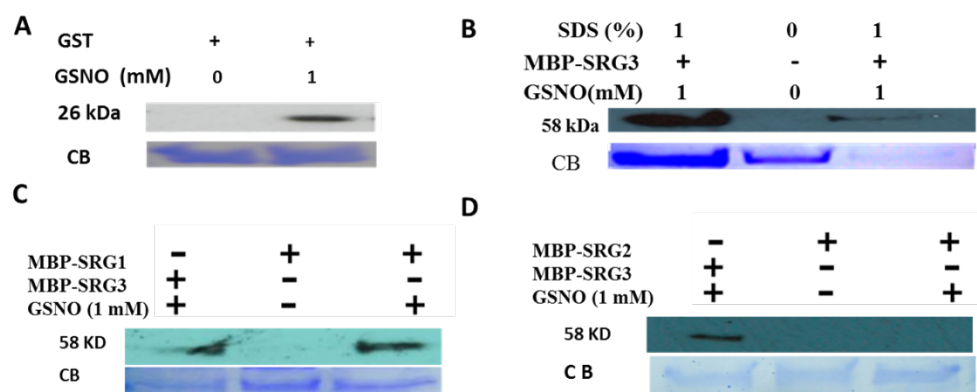


Figure 4.5 S-nitrosylation of SRG1 and SRG3 *in vitro*.

The purified proteins Glutathione S-transferase (GST) (A), MBP-SRG3 (B), MBP-SRG2 (D) and MBP-SRG1 (C) were subjected to biotin switch assay for S-nitrosylation assay. The proteins were detected by western blot with anti-biotin antibody with NO donor GSNO. CB, coomassie blue staining.

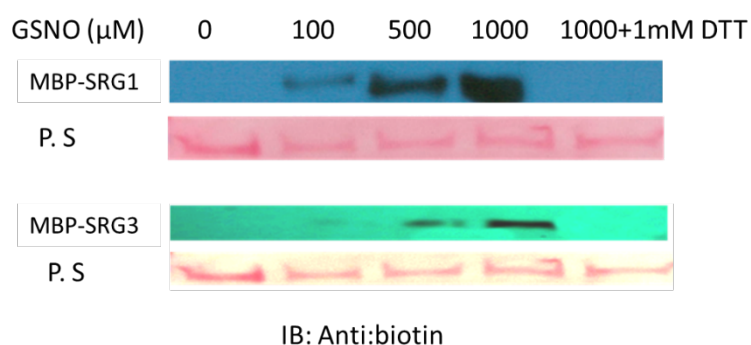


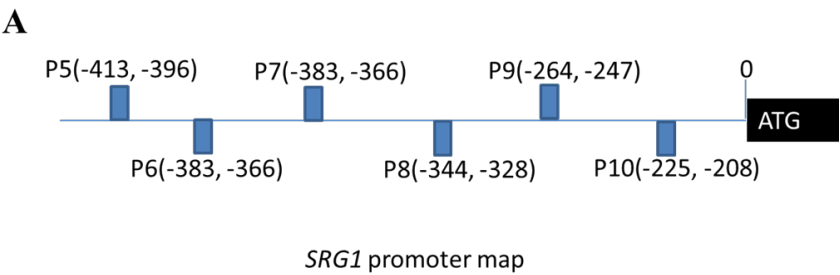
Figure 4.6 S-nitrosylation of SRG1 and SRG3 is GSNO concentration dependent and reversed by dithiothreitol treatment.

The recombinant protein MBP-SRG1 (A) and MBP-SRG3 (B) were treated with increasing concentrations of GSNO with or without dithiothreitol (DTT) indicated in the figure. After treatment the samples were subjected to biotin switch assay and the total proteins were separated by SDS-PAGE. The S-nitrosylation of SRG1 and SRG3 were determined by western blot against anti-biotin antibody. Total protein was stained with ponceau stain. P. S, ponceau stain.

4.5 SRG1 exhibits DNA binding activity and its DNA binding activity is regulated by NO

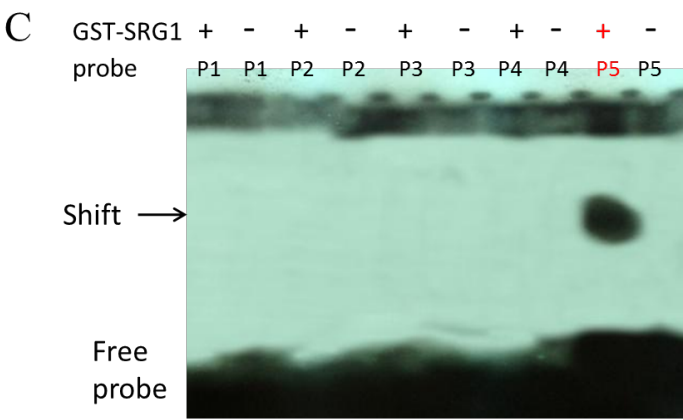
Research has shown that C2H2- type zinc finger proteins have a conserved QALGGH motif in the zinc finger domain and could bind to target promoters with a AG/CT motif ([Fukushima et al., 2012](#)). To examine whether SRGs display the DNA binding activity, the selected oligos with an AC/GT sequences generated randomly or taken from the *SRG1* promoter sequence (Figure 4.7A) were used to test the DNA binding activity of SRG1. Electrophoretic mobility shift assay (EMSA) was carried out and results showed that among the selected 10 oligos, two of them could be recognized by SRG1, when the AGT sequences in the oligo was separated by 4 or 6 bases, respectively. This result is in agreement with previous publication ([Fukushima et al., 2012](#)). In order to test whether AGT is the core sequence for SRG1 binding, the AGT sequences were mutated to AAA in the targeted oligos. These mutated oligos could not be binded by SRG1 (Figure 4.8B). This data suggested that the AGT sequence is required in the tested oligos for SRG1 recognition.

Predictions from the animal field have suggested that the zinc finger domain might be a possible target of S-nitrosylation, with this PTM possibly destroying its DNA binding structure ([Kröncke, 2001](#), [Sha and Marshall, 2012](#), [Tavares et al., 2014](#)). By extension, SRG1 could also be a possible target for S-nitrosylation *in vitro*. Figure 4.8 shows that application of the NO donor, GSNO, reduces the amount of SRG1-DNA complex formation, suggesting NO could blunt SRG1 DNA binding activity. Our results therefore provide the direct evidence that NO could impair zinc finger-mediated DNA binding activity, possibly by forming an SNO with a reactive cysteine group.



B

P1: GGGGACTGATCCAGT
P2: GGGGAGTGATCCACT
P3: GGGGAGTGCATCGCATCACT
P4: GGGGAGTGCATCGCATCAGT
P5: GGGGAGTCTCATAAGTGTTAC



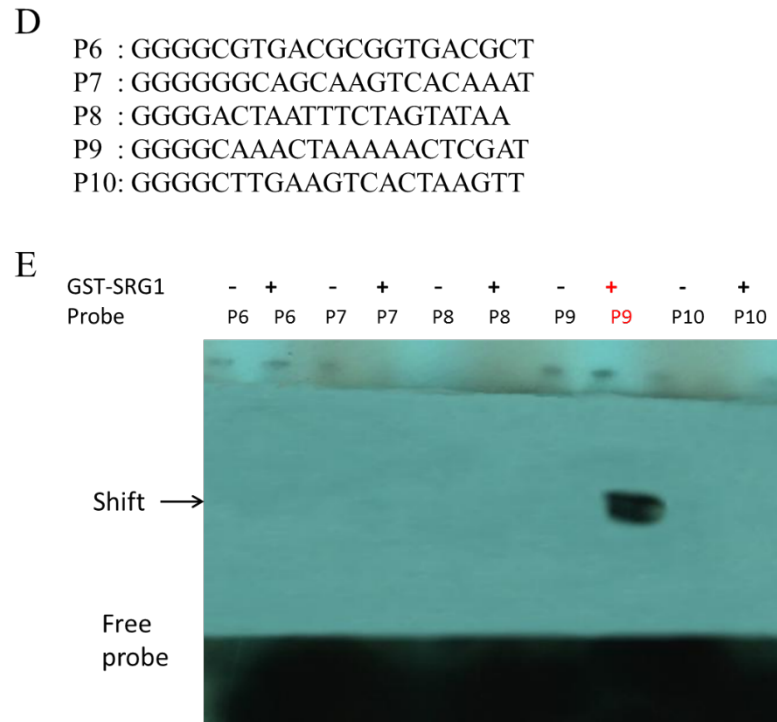


Figure 4.7 Recombinant MBP-SRG1 binds to AGT/AGT sequences separated by 4 or 6 nucleotides.

The probes P1-P4 were generated randomly with ACT or AGT sequences, and the probes P5-P10 were obtained from *SRG1* promoter and its location were labeled in *SRG1* promoter (A). Oligonucleotide sequences were presented in (A) and (C). Gel shift assays were used to determine the possible interaction between recombinant GST-SRG1 and the selected oligonucleotide sequences (D, E). A DNA shift, indicating GST-SRG1 binding to a given oligonucleotide is marked by a black arrow. -, without recombinant protein. +, with recombinant protein.

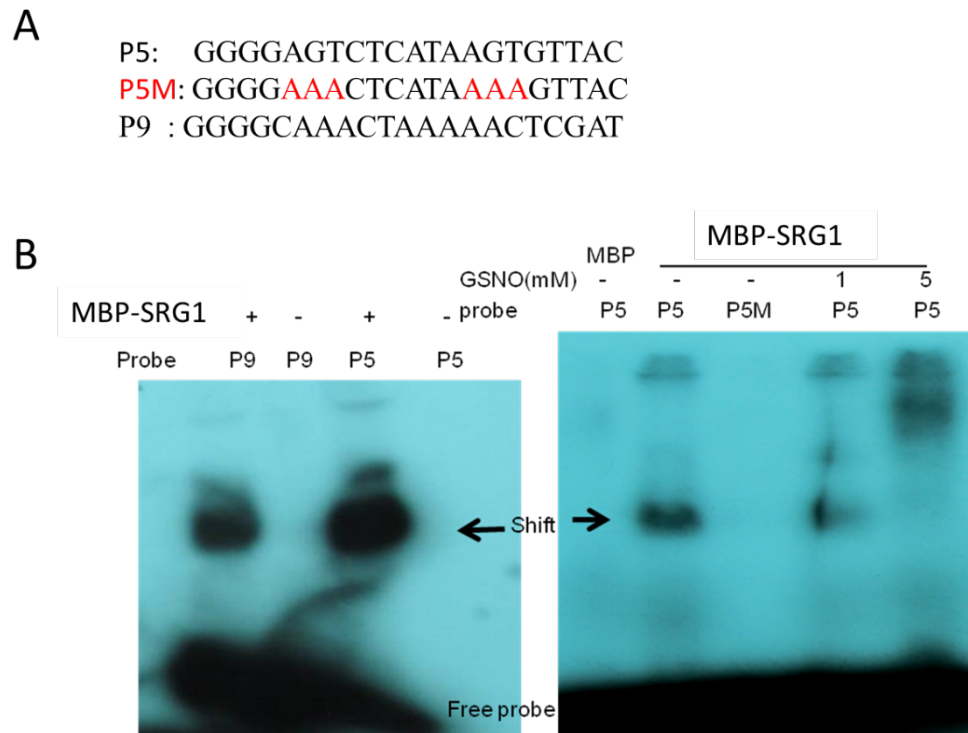


Figure 4.8 NO regulates DNA binding activity of SRG1 *in vitro*.

Oligonucleotide sequences employed are presented in (A). The AGT or mutated oligonucleotides were highlighted in red. Gel shift assays were used to determine the interaction of recombinant MBP-SRG1 and the selected oligonucleotide. In the presence of MBP-SRG1 (+) or absence (-). Oligonucleotide shift is marked by a black arrow.

4.6 SRG1 and SRG3, but not SRG2, could be recognized by *Arabidopsis* MAPK3 and MAPK6

In plants, MAPKs play critical roles in plant defence by activating or inactivating downstream targets through phosphorylation ([Asai et al., 2002](#), [Meng et al., 2013](#), [Meng and Zhang, 2013](#)). From sequence analysis, there is a putative MAPK docking site at the C-terminus of SRGs. Also, a related protein of SRGs, PtiZFP1, found in poplar, could be bound by MAPK3 and MAPK6 in response to stress, suggesting it might be a substrate for these MAPKs ([Hamel et al., 2011](#)). Moreover, MAPK3 and MAPK6 are important regulators in plant immunity ([Meng and Zhang, 2013](#)). As transcription factors, SRG proteins are likely to be regulated very precisely in space and time. Therefore, we examined whether SRGs could interact with MAPK3 and

MAPK6.

We amplified the coding sequences of *MAPK3* and *MAPK6* (Figure 4.9) from *Arabidopsis* Col-0 cDNA and then *MAPK3* and *MAPK6* were cloned into pDONR221 by the BP reaction (Invitrogen, USA) for further experiments.

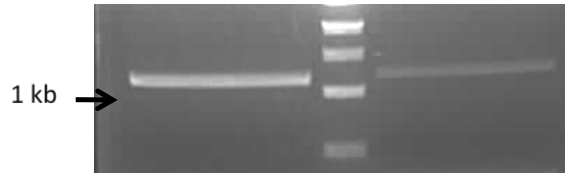


Figure 4.9 Amplification of the CDS of *MAPK3* and *MAPK6* from *Arabidopsis* Col-0 cDNA.

The full length of coding sequences (CDS) of *MAPK3* (first lane) and *MAPK6* (third lane) were amplified by PCR from Col-0 cDNA. A DNA marker was loaded in the second lane and 1 kb was highlighted by a black arrow.

4.6.1 SRG1 and SRG3 can interact with MAPK3 and MAPK6 in yeast

Firstly, we test the interactions between SRGs and MAPK3 or MAPK6 by a yeast two-hybrid assay (Y2H). The *SRGs* in pDNOR221 were cloned into pDEST32 to generate SRGs fused to the GAL-4 binding domain (SRGs-BD) which served as bait proteins. On the contrary, the *Arabidopsis* *MAPK3* and *MAPK6* were cloned into pDEST22 to fuse with the GAL4-activation domain (AD) which served as the prey protein. The pDEST32 and pDEST22 were used as the bait and prey vector control. The recombinant plasmids with prey and bait were co-transformed into yeast strain AH109. The successful transformation on the selected medium synthetic drop out plates without leucine and tryptophan (SD-T-L) were genotyped and tested on synthetic drop out plates without leucine, tryptophan and histidine (SD-T-L-H), and plus 3-amino-1,2,4-triazole (3-AT). Figure 4.10 showed that co-transformation of MAPK3 or MAPK6 and SRG1 or SRG3 can growth on SD-T-L-H medium, but the yeast cells with co-transformation of SRG2 and MAPK3 or SRG2 and MAPK6 could not, which suggested that SRG1 and SRG3 could interact with MAPK3/6, but SRG2 might not. To verify the interactions, 5-bromo-4-chloro-3-indolyl alpha-D-

galactopyranoside (X-alpha-gal) was added to SD-T-L-H+3-AT media and the interactions between SRG1 and MAPK3/6, SRG3 and MAK3/6 were confirmed by the formation of blue colonies. These results suggested SRG1 and SRG3 could interact with MAPK3 and MAPK6 in yeast, but SRG2 did not interact with MAPK3 or MAPK6 in yeast.

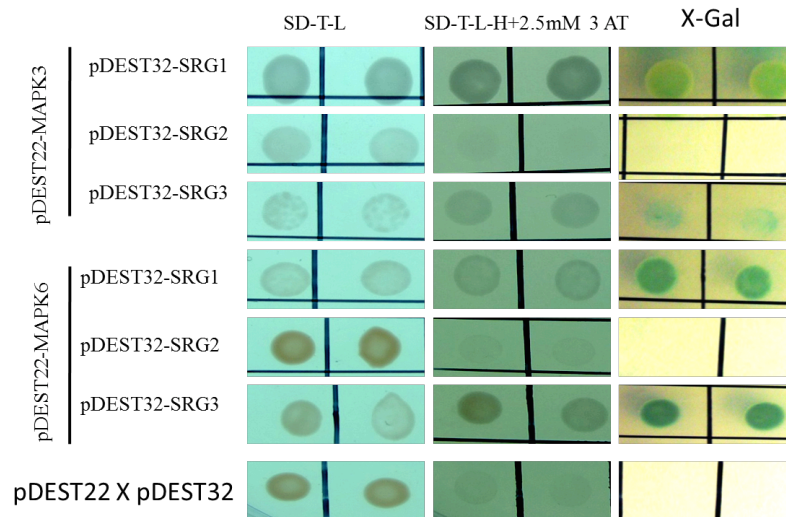


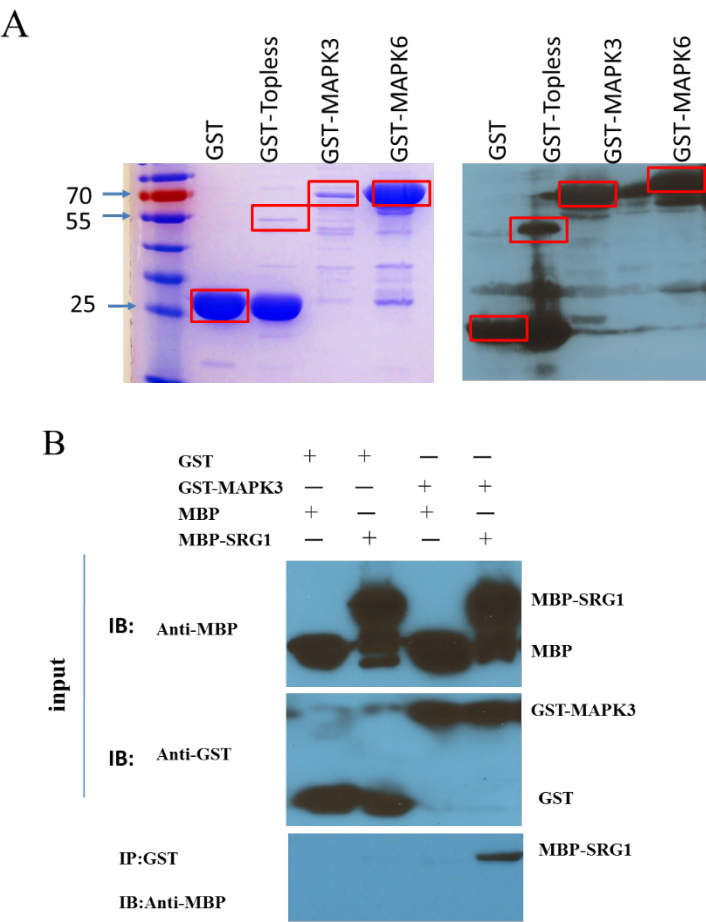
Figure 4.10 characterization of the interactions between SRG and MAPK3/6.

AH109 cells were cotransformed with pDEST22 or pDEST-MAPK3/6 and pDEST32 or pDEST32-SRGs. The transformants were genotyped and spotted on control medium (SD-T-L) and selection medium (SD-T-L-H, 2.5 mM 3-AT) to monitor *HIS* reporter gene. The confirmation of interactions between SRGs and MAPK3/6 was assayed using X-alpha-gal by checking *lacZ* activity. The empty vectors pDEST32 and pDEST22 were used as the negative control.

4.6.2 SRG1 and SRG3 could interact with MAPK3 and MAPK6 *in vitro*

To further examine the interactions between SRGs and MAPK3/6, a pull-down assay was performed *in vitro*. Firstly, Glutathione S-transferase (GST), GST-MAPK3 and GST-MAPK6 were expressed and purified from *E. coli* and purified proteins were confirmed by western blot with anti-GST antibody (Figure 4.11-A). Subsequently, purified GST, GST-MAPK3 or GST-MAPK6 was immobilized on 4B beads and tested for their ability to pull-down MBP, MBP-SRG1 and MBP-SRG3. Figure 4.11-B shows MBP-SRG1 could pull-down GST-MAPK3 and GST-MAPK6

(Figure 4.11-C), whereas MBP could not pull-down GST, GST-MAPK3 or GST-MAPK6. Obviously, MBP-SRG1 could also not pull-down GST. Meanwhile, MBP-SRG3 could pull-down GST-MAPK3 (Figure 4.11-D) and GST-MAPK6 (Figure 4.11-E), but not GST. These results support the results in yeast suggesting that SRG1 and SRG3 could interact with MAPK3/6.



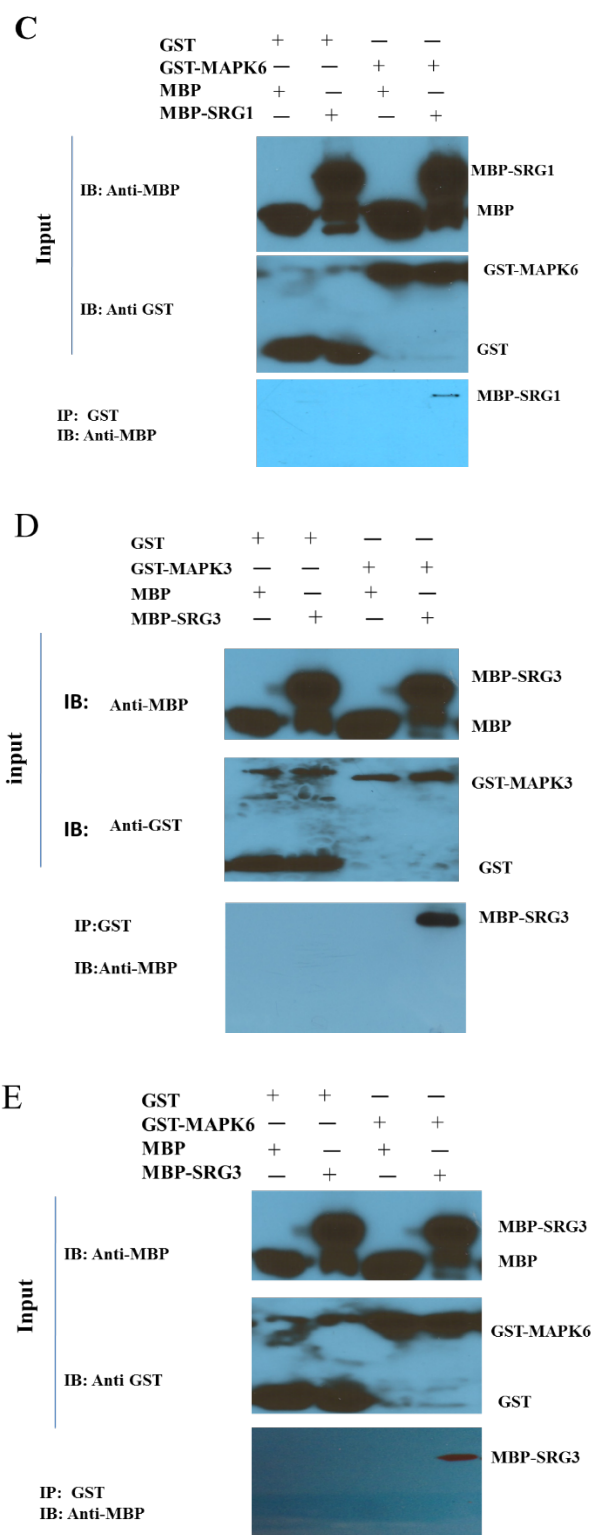


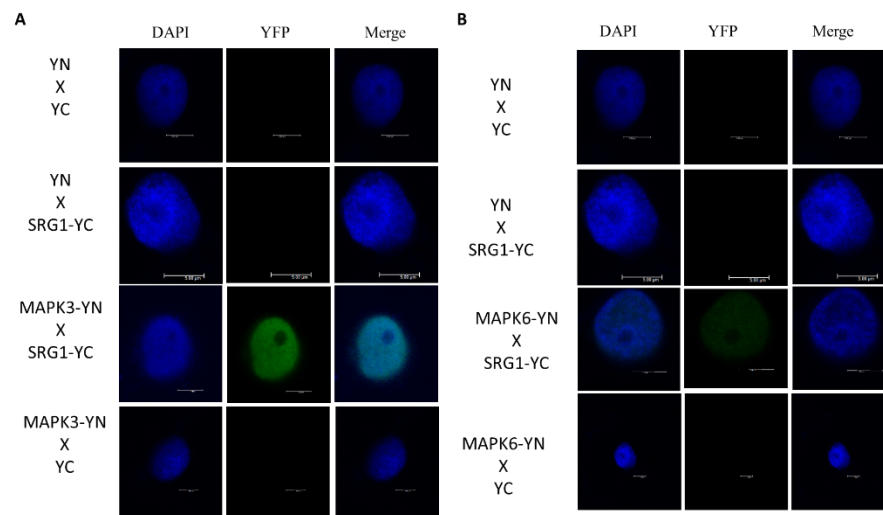
Figure 4.11 SRG1/3 interacts with MAPK3/6 *in vitro*.

A, Purified recombinant proteins, previously expressed in *E. coli*, were separated on SDS page and monitored by western blotting using an anti-GST antibody. A GST pull down was carried out

to determine the possible interaction between SRG1 and MAPK3 (B), SRG1 and MAPK6 (C), SRG3 and MAPK3 (D) and SRG3 and MAPK6 (E). Input indicated the proteins used for the given pull down assay. The protein from input or GST pull down samples was subjected to western blotting against an anti-GST or anti-MBP antibody. IB, immunoblotting, IP, immunoprecipitation. The target protein was labeled in the images.

4.6.3 SRG1 and SRG3 can interact with MAPK3 and MAPK6 in plants

To further confirm the interactions between SRG1/3 and MAPK3/6 in yeast and *in vitro*, a bimolecular fluorescence complementation (BiFC) assay was conducted in tobacco leaves to further analyze the interactions in plants. SRG1/3 were each fused to the C-terminal-encoding portions of YFP (SRG1-YC, SRG3-YC), whereas the N-terminus of YFP was fused to MAPK3/6 (MAPK3/6-YN). The YFP signal could be detected in the co-transformation of SRG1/3 with MAPK3/6 (Figure 4.12) in tobacco leaves 36 hpi, while the YFP signal in the combinations of Y-N or MAPK3/6 with Y-C and SRG1/3-YC with YN was not detected. Thus, these data suggest that SRG1/3 does interact with MAPK3/6 in plants.



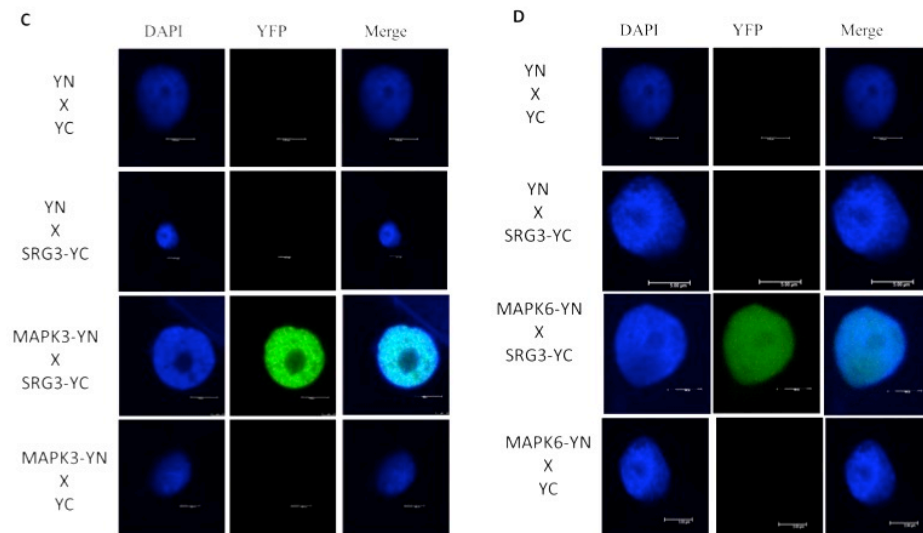


Figure 4.12 SRG1/3 interacts with MAPK3/6 *in vivo*.

The pairs of recombinant plasmids; YN (YFP-N-terminus) and YC (YFP-C-terminus) were transformed into tobacco leaves by *Agrobacterium* GV3101. YFP signals were monitored after 36 hours post infiltration. The interaction between SRG1 with MAPK3 (A), SRG1 and MAPK6 (B), SRG3 and MAPK6 (C) and SRG3 and MAPK6 (D) were assessed. The nucleus was stained by DAPI (4,6-diamidino-2-phenylindole). YFP, yellow fluorescent protein.

4.7 SRG1 and SRG3 levels are controlled by the 26S proteasome

Transcription factors regulate downstream signalling by activating or inactivating transcription of its target genes. Typically, transcription factors are precisely controlled by posttranslational modifications, such as phosphorylation ([Pitzschke, 2015](#)) or ubiquitination ([Devoto et al., 2003](#)). Previous research has suggested that proteins with an EAR motif and MAPK docking site might be subject to degradation by the 26S proteasome system ([Hamel et al., 2011](#), [Hamel et al., 2012](#)).

We found SRG1/3 could interact with MAPK3/6 and also that these transcription factors have a putative EAR motif. To explore whether SRG1/3 were turned over by the 26S proteasome, cell-free degradation assay ([Wang et al., 2009a](#)) was carried out with recombinant protein MBP-SRG1/3. Specifically, total proteins were extracted from Col-0 plants after treatment with H₂O₂, which has been shown to activate MAPK3/6 in *Arabidopsis* ([Rentel et al., 2004](#)). Figure 4.13-A shows that MBP-SRG1

was degraded from 10 minutes and was almost totally depleted after 60 minutes. Similarly, MBP-SRG3 was also degraded. On the contrary, the MBP was stable. The data therefore suggested that SRG1 and SRG3 were relatively unstable. ATP is required by the 26S proteasome system for degradation of target proteins (Wang, 2009). In the absence of ATP, the stability of MBP-SRG1 was enhanced. Further, MG132, an inhibitor of the 26S proteasome system, delayed the degradation of SRG1 (Figure 4.13-B), indicating SRG1 could be subjected to degradation by 26S proteasome.

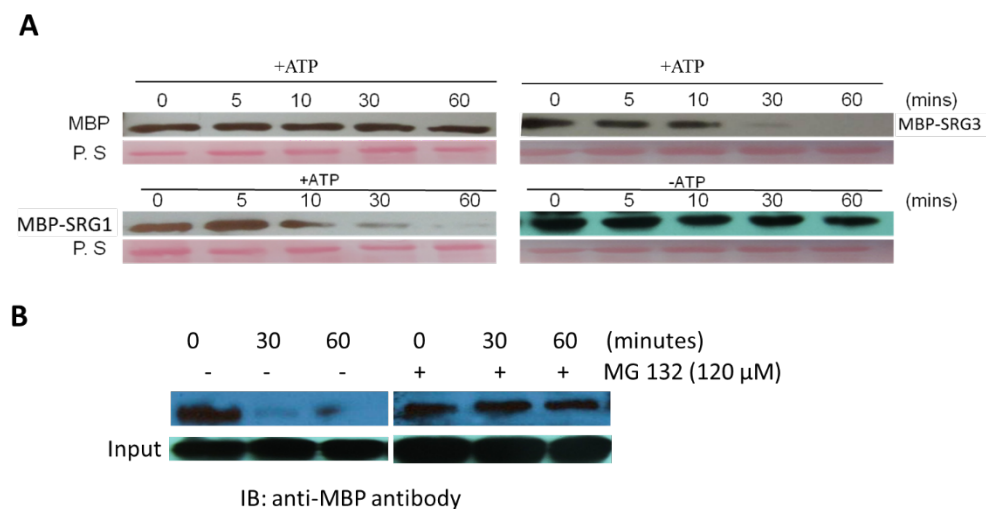


Figure 4.13 Degradation of SRG1 and SRG3.

A, Cell free degradation assay was conducted to monitor the stability of recombinant MBP, MBP-SRG1 and MBP-SRG3 in the presence or absence of ATP. B, The 26S proteasome inhibitor MG132 was employed to check whether it could blunt the degradation of MBP-SRG1. Incubation times were indicated and the concentration of MG132 is shown. Total proteins were indicated with P. S. P.S, ponceau stain.

4.8 SRGs function as transcriptional repressors

SRGs contain an EAR-motif like sequence in the C-terminus, this domain has been predicted to function as a transcriptional repressor ([Kagale and Rozwadowski, 2011b](#)). To clarify if the EAR-motif can function as a repressor when in the context of SRGs, an *Arabidopsis* protoplast assay was conducted to determine the transcriptional

activity of SRGs. In this experiment, the full length sequence of SRGs were fused to the C-terminus of the *GAL4-BD* (Frunghillo et al.) effector plasmid, with their expression driven by the *CaMV 35S* promoter (*35S:GAL4-SRG1*, *35S:GAL4-SRG2*, *35S:GAL4-SRG3*) to generate the effector vectors. Whereas, for the reporter plasmid, with 5 copies of the GAL4 was fused to the luciferase (LUC) reporter (Guo et al., 2013). SRG transcriptional activity was tested after introducing a given effector plasmid along with the reporter plasmid into *Arabidopsis* protoplasts. For an internal control, a plasmid with a *renilla* luciferase gene driven by the *CaMV 35S* promoter was co-transformed for normalization. Figure 4.14 shows that LUC activity was reduced ~50% by the presence of the SRG-GAL4 proteins. This data implies that SRG proteins may function as transcriptional repressors *in planta*.

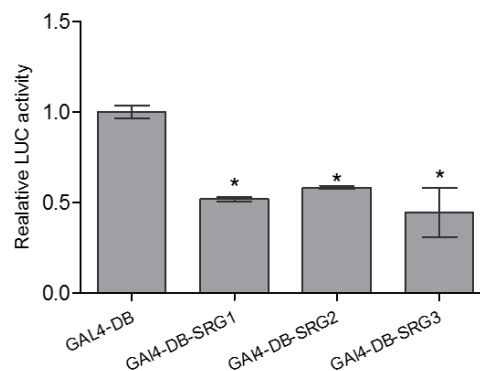


Figure 4.14 SRG proteins function as transcriptional repressors.

LUC activities were determined after co-transformation of *Arabidopsis* protoplasts with *GAL-4DB* (GAL4) or *GAL-DB-SRG* (GAL4-SRG) and the *GAL4::LUC* reporter. The LUC activity in each assay was normalized relative to that of the internal control and then normalized to GAL4. Asterisks indicate statistically significant differences compared with GAL4-DB. (*, $P < 0.05$)

4.9 SRG1 interacts with TOPLESS

Previous data has suggested that the EAR motif might interact with TOPLESS to form a transcriptional repressor complex (Kagale and Rozwadowski, 2011a). Thus, SRG1 was assessed for the possible interaction with TOPLESS. The N-terminus of

TOPLESS, containing its protein interaction motif, was utilised in Y2H to test the binding to SRG proteins. Figure 4.15-C showed that SRG1 could interact with TOPLESS in a Y2H assay. GST pull down assay (Figure 4.15-A) and BiFC data (Figure 4.15-C) confirmed the Y2H data. Taken together, SRG1 could interact with TOPLESS.

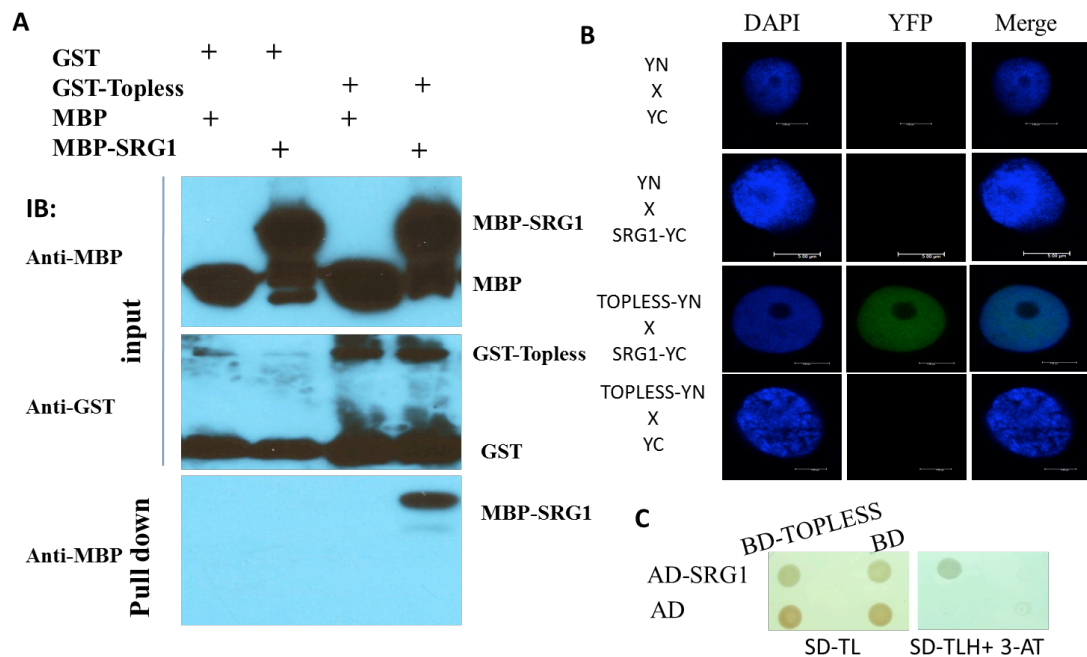


Figure 4.15 SRG1 interacts with TOPLESS *in vitro* and *in vivo*.

(A) GST pull-down assay of recombinant GST-TOPLESS binding to MBP-SRG1. MBP-SRG1 with GST and MBP with GST-Topless were used as negative controls. (B) BiFC assays with SRG1 and TOPLESS in tobacco leaf epidermal cells. Images were captured 36 h after transient expression under confocal microscope. YN and YC vectors were used as negative controls. (C) Yeast two-hybrid assays showing the interaction between SRG1 and TOPLESS. Yeast growth on dropout selection medium (SD-T-L-H plus 2.5 mM 3-AT) indicates interaction between the SRG1 and TOPLESS.

4.10 Discussion

S-nitrosylation has emerged as a key signalling mechanism for the transfer of NO bioactivity in plant development and stress response (Yu et al., 2012, Yu et al.,

2014). We have identified a sub group of ZF TFs that can be induced by NO at the transcriptional level. Here we show SRG1 and SRG3 could also be modified at the posttranslational level by S-nitrosylation. In recent years, some important proteins involved in plant immunity have been identified as targets of NO such as NADPH oxidase (RBOHD) (Yun et al., 2011) and NPR1 (Tada et al., 2008). Specifically, RBOHD functions as the most important reactive oxygen intermediates synthesis enzyme. S-nitrosylation of RBOHD at Cys⁸⁹⁰ blocked its binding to cofactor flavin adenine dinucleotide (FAD), resulting in loss of its ability to synthesize reactive oxygen intermediates (ROI). NPR1, an important regulator of SA-dependent gene expression, was regulated by NO upon pathogen challenge. In unchallenged cells, NPR1 is primarily located in the cytoplasm in the form of an oligomer established by intermolecular disulphide bonds. In the presence of pathogens, oligomers are converted to monomers, which is mediated by S-nitrosylation at Cys¹⁵⁶, the monomers then moved to nucleus to enhance the target genes expression, such as pathogenesis-related genes (*PRs*). Additionally, SABP3 (Wang et al., 2009b) and TGA1 (Lindermayr et al., 2010) also was modified by S-nitrosylation during plant immunity. Our results demonstrated SRG1 and SRG3 could be S-nitrosylated, but not the SRG2, suggesting NO may selectively modify these proteins despite their high sequence similarity.

It is reported that NO not only regulates protein activity at the posttranslational level but also modulates gene expression at the transcriptional level in animals (Sha and Marshall, 2012). In this context, NO could regulate the DNA binding function or transcriptional activity of TFs to reprogramme gene expression by S-nitrosylation of cognate highly reactive cysteine thiols. However, how NO regulates gene expression at the transcriptional level is poorly understood in plants.

Recently, AtMYB30 was found to have its DNA binding activity blunted in the presence of NO, maybe due to a redox-modification *in vitro* (Tavares et al., 2014). Although it is still unclear whether S-nitrosylation of AtMYB30 represses its DNA binding activity *in vivo*, it promoted researchers to consider the possibility that NO could also regulate the transcriptional activity of transcription factor by S-nitrosylation in plants. Our data suggests that NO could regulate the DNA binding

activity of a zinc finger containing transcription factor SRG1 by S-nitrosylation and provides the first direct evidence that NO could blunt the DNA binding activity of zinc finger proteins in both plants and animals. By extension, it is possible that C2H2-type zinc finger containing transcription factor SRGs might serve as global regulators of NO function in plants. SRGs were NO inducible and they contain conserved cysteines in the zinc finger domain. Importantly, zinc finger containing transcription factors are one of the biggest transcription factor families and the cysteines in the zinc finger domain are sensitive to S-nitrosylation. These results suggest that NO may reprogramme gene expression during plant immunity by regulating the transcriptional activity of SRGs by S-nitrosylation.

SRGs possess a putative plant specific EAR motif, LxLxL, located at the C-terminus of SRGs ([Ciftci-Yilmaz et al., 2007](#)), which functions as a repressor of transcription. As expected, SRGs showed transcriptional repression activity in an *Arabidopsis* protoplast transient repression activity assay. Furthermore, EAR-containing transcriptional repressors are proposed to become an important new theme in plant development and stress responses via the reprogramming of gene expression ([Kazan, 2006](#), [Kagale and Rozwadowski, 2011b](#)). Recently, two C2H2-type zinc finger proteins DAZ1/DAZ2, EAR motif-containing transcription factors, were identified to regulate cell division during the G2- to M-phase transition in *Arabidopsis* by repressing the expression of *DUOI* (DUO POLLEN1), which is the male germline-specific transcription factor involved in regulation of G2 ([Borg et al., 2014](#)). Meanwhile, the important regulator NINJA, the novel interactor of JAZ, functions as a transcriptional repressor of JA signaling due to its EAR motif ([Pauwels et al., 2010](#)). In this context, the EAR motif in NINJA interacts with the co-repressor TOPLESS to form a repressor complex.

It has also been reported that SRG2 and SRG3 were involved in drought and salt responses and might repress a repressor of related defence genes ([Rizhsky et al., 2004](#), [Davletova et al., 2005](#), [Ciftci-Yilmaz et al., 2007](#), [Rai et al., 2014](#)). However, it is not clear how SRGs act as a repressor to regulate gene expression in response to stress. In our study, we found SRGs could interact with TOPLESS *in vitro* and *in vivo*, suggesting that SRGs may recruit the corepressor TOPLESS to mediate

transcriptional repression activity. Research has shown that the EAR motif normally interacts with a co-repressor, such as TOPLESS or SAT18, to assemble the repressor complex to mediate repression ([Kagale and Rozwadowski, 2011b](#)). Whether this formation is required for repression activity in SRGs still needs further experimentation.

Posttranslational modification of repressor function is a common way for plants to modulate EAR-mediated suppression, such modifications might include phosphorylation or ubiquitination. These modifications might influence the interactions between a repressor and its partner, the turnover of the repressor, or its repression activity. Interestingly, we found that S-nitrosylation of SRG1 could blunt its DNA binding activity, which provides a new mechanism to control EAR repression activity in plants.

In addition to the EAR motif, there is a MAPK docking site, which may be recognized by MAPKs. Experiments investigating protein interactions were conducted by BiFC, Y2H and pull down assays and the results showed SRG1 and SRG3 could interact with MAPK3 and MAPK6. Similarly, in POLAR, an EAR-motif containing zinc finger protein, interacts with MAPK3/6, subsequently this zinc finger protein was phosphorylated by MAPK3/6 and subjected to 26S proteasome degradation ([Hamel et al., 2011](#)). MG132, a 26S proteasome specific inhibitor, was employed and found to blunt SRG1 degradation. Collectively, our results suggest that SRG1 might be phosphorylated by MAPK3/6, which subsequently primes degradation through the 26S proteasome, which could then remove the transcriptional activity of SRGs through their turnover.

Chapter

5 Characterization of the roles of *SRGs* in plant immunity

5.1 Introduction

Plants have developed complicated strategies to defend against pathogens. Following pathogen infection, the nitrosative burst is a conspicuous feature during plant immunity ([Yu et al., 2014](#)). Consequently, NO is a key redox signal molecule and plays important functions in plant immunity. In response to pathogens, NO can modulate protein activity by S-nitrosylation ([Feechan et al., 2005](#), [Bellin et al., 2013](#)). NO can also reprogramme global transcription ([Delledonne et al., 1998](#)). Although NO has recently been proposed to S-nitrosylate the basic leucine zipper transcription factor, TGA1 ([Lindermayr et al., 2010](#)), which regulates a sub-set of SA- response genes, little is known about how NO reconfigures global gene expression.

In order to dissect the mechanism of NO signalling during the immune response, we identified transcription factor *SRGs* (SNO-Regulated Genes) in *Arabidopsis* and are exploring their possible roles in plant immunity. In previous chapters we have demonstrated that *SRG* genes could be induced by pathogens as well as NO, and *SRG1*/*SRG3* could be modified by NO *in vitro*. Furthermore, S-nitrosylation of *SRG1* was found to influence its DNA binding activity. The detailed roles of *SRGs* in the plant defence response therefore warrant investigation. In this chapter, we describe the identification of *SRG Arabidopsis* T-DNA insertion lines and generate lines overexpressing *SRG*, to analyse the function of *SRG* genes in plant immunity.

5.2 Identification and characterization of *srg* T-DNA insertion lines

In order to dissect the roles of *SRG* genes in plant immunity, T-DNA insertion lines were ordered from The Nottingham Arabidopsis Stock Centre (NASC). The T-DNA insertion in the 5'UTR region of *srg1* (SALK_119663), the promoter region of *srg2* (GABI_404D05) and exon of *srg3* (SAIL_1213_C07) were obtained for further experiments. According to the information in Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu/tdnaprimers.2.html>), the primers for *srg* mutant

genotyping were designed and the primer LB for the left T-DNA border was also used. The genomic DNA from the ordered mutant lines was extracted using the CTAB method and PCR was conducted with these three primers. The PCR product was loaded on agarose gels and figure 5.1 showed the PCR results. Specifically, wild type Col-0 should give only a product of ~1 Kb, homozygous T-DNA insertion line should give a ~ 0.5 kb product (marked by double asterisks in figure 5.1), while for heterozygous lines two bands (~0.5 kb and ~1 kb, respectively) are expected (marked by signal asterisks in figure 5.1). Based on the PCR results, we identified some homozygous T-DNA insertion lines of *srg1* (Figure 5.1A), *srg2* (Figure 5.1B) and *srg3* (Figure 5.1C) and seeds from these lines were collected for further experiments.

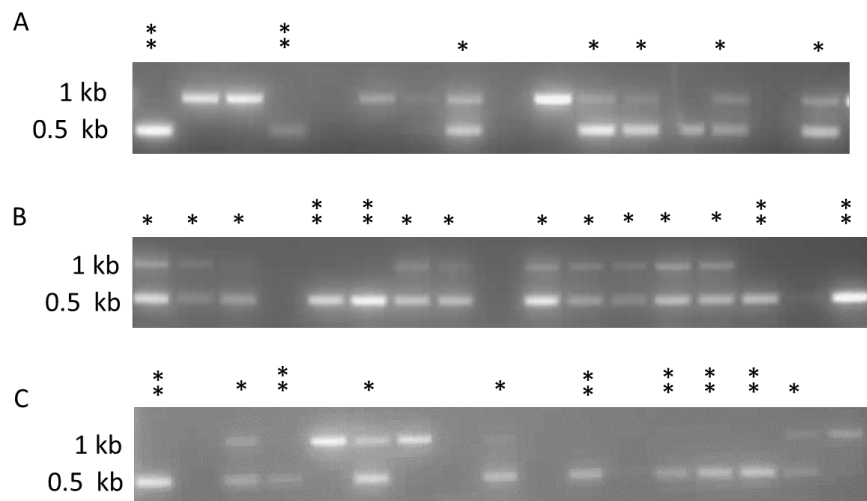


Figure 5.1 Genotyping for *SRG* T-DNA insertion lines.

Three primers including gene specific primers and T-DNA insertion LB board primer were employed for genotyping to identify homozygous T-DNA insertion mutant lines based on PCR. The genomic DNA was extracted from the T-DNA insertion lines of *srg1* (A), *srg2* (B) and *srg3* (C) from NASC. The PCR was performed and the PCR products were separated on agarose gels. The non-insertion lines only give a single product of about 1 kb which is the same with Col-0. The homozygous lines give a single product of ~ 500 bp which was highlighted by two asterisks (**) and the heterozygous lines give two bands, one band (~1 kb) from wild type and another (~500 bp) from the resulting T-DNA insertion, as indicated by asterisk (*) in the pictures. 0.5 kb and 1 kb size fragments in DNA ladder are highlighted.

The confirmed homozygous *srg* plants were grown under short day conditions. The growth phenotype was observed and interestingly at 6-week-old *srg* lines were found to be visibly larger than Col-0 plants (Figure 5.2).

For further confirmation of T-DNA insertion mutant lines, *SRG* mRNA level in these lines was determined. RNA was extracted from 4-week-old plants and a qRT-PCR assay was carried out to analyse the transcripts level in these lines. The *SRG* RNA level in these *srg* mutants was reduced significantly compared with that in Col-0 plants (Figure 5.2). T-DNA insertion resulted in reduction of transcripts of *SRG*, despite the T-DNA insertion being in the promoter region. Of these candidate *srg* mutant lines, *SRG3* expression levels in *srg3* were only reduced to ~50% relative to Col-0. These results confirmed that T-DNA insertion mutants knock down *SRG* transcript levels.

In order to quantify the effects of T-DNA insertion on growth, fresh weight of individual lines were quantified. Figure 5.3 shows *srg* mutations promoted *Arabidopsis* growth compared with Col-0, which is consistent with the phenotype as shown in figure 5.2. Statistical analysis showed *srg1* and *srg3* were apparently heavier than Col-0 (Figure 5.3). Moreover, we also quantified the leaf area and found the leaf area of *srg* lines was significantly larger than Col-0 statistically. Collectively, these results demonstrate that *SRGs* negatively regulate *Arabidopsis* growth.

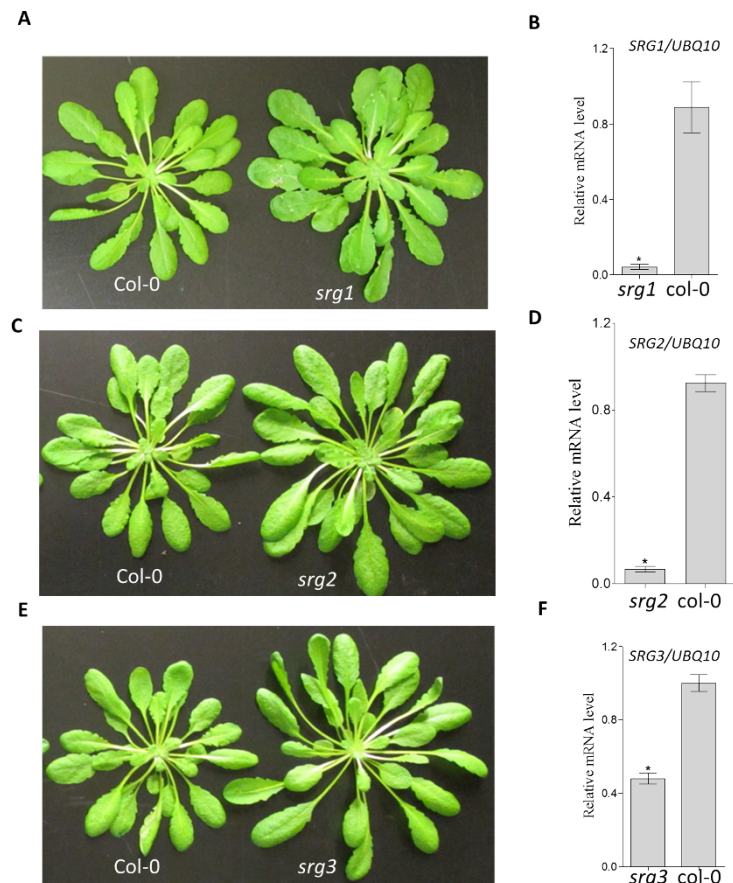


Figure 5.2 Phenotype and relative RNA level analysis of *srg* T-DNA insertion lines.

The Col-0 and T-DNA insertion lines were soil-grown under short day conditions and the 6-week-old Col-0, *srg1* (A), *srg22* (C) and *srg3* (E) plants were photographed. Correspondingly, RNA was extracted from 4-week-old Col-0 and *srg* mutant lines, separately and qRT-PCR was performed to check the RNA level in these lines. The level of mRNA of *srg1* (B), *srg2* (D) and *srg3* (F) was compared with that in Col-0. The transcripts of *SRG* in Col-0 was normalized as 1.0. *Arabidopsis UBQ10* was used as the internal control for qRT-PCR. Error bars represent the SD from at least 3 independent biological replicates. Asterisk indicates the significant difference between mutant *srg* lines and Col-0 determined by student *t* test with *P* value <0.05 and triple asterisks indicate *P* value <0.01.

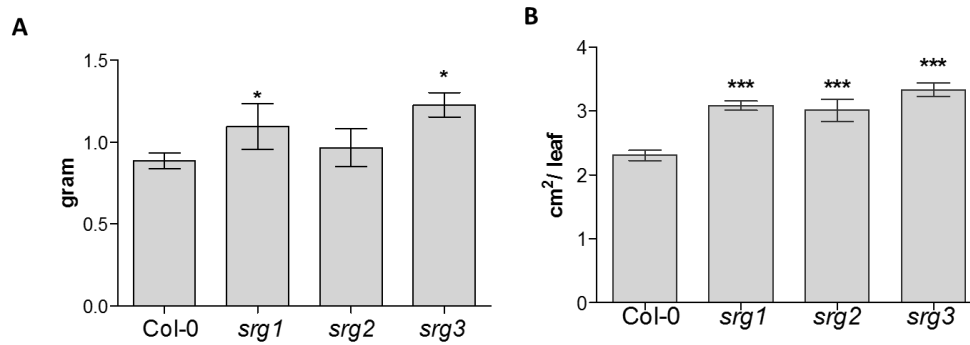


Figure 5.3 *Arabidopsis srg* mutant lines exhibit enhanced growth.

(A) The fresh weight and (B) the leaf area of Col-0 and *srg* homozygous mutant lines from 6-week-old plants under short day conditions were recorded. Error bars represent SD and the mean value is derived from at least 8 independent lines. Asterisk indicates the significant difference between mutant *srg* lines and Col-0 determined by student *t* test with $P < 0.05$ and triple asterisks indicate $P < 0.001$.

5.3 *SRG* over-expression lines and their phenotypic analysis

To gain further insight of *SRG* genes in plant immunity, we generated transgenic *Arabidopsis* lines that express *SRG* under the control of the CaMV 35S promoter. *SRG1*, *SRG2* and *SRG3* were subcloned into the binary vector pGWB11 by the Gateway system (Invitrogen, USA), separately, and the sequenced plasmids were transformed into Col-0 by floral dip method ([Zhang et al., 2006](#)). Subsequently the resulting seeds were screened on 1/2 MS with kanamycin, as pGWB11 contains kanamycin resistance gene as a selection marker. Selected plants were confirmed by PCR with sequencing primers for the vector pGWB11. As shown in figure 5.4, genomic DNA extracted from Col-0 and *SRG* overexpressing transgenic plants were used for genotyping. These transformants gave a product of ~0.5 kb, whereas Col-0 did not, which confirmed the insertion. Representative T3 homozygous line with a single insertion overexpressing *SRG* (*SRG-OX*) were used for further experiments. *SRG-OX* plants were soil-grown under short day conditions and these *SRG-OX* plants were affected in their growth (Figure 5.5a) compared with Col-0. *SRG2-OX* significantly inhibited plant growth and generated curved leaves (Figure 5.5B). Further, *SRG3-OX* plants exhibited the most marked growth reduction and leaves

were significantly narrowed (Figure 5.5C).

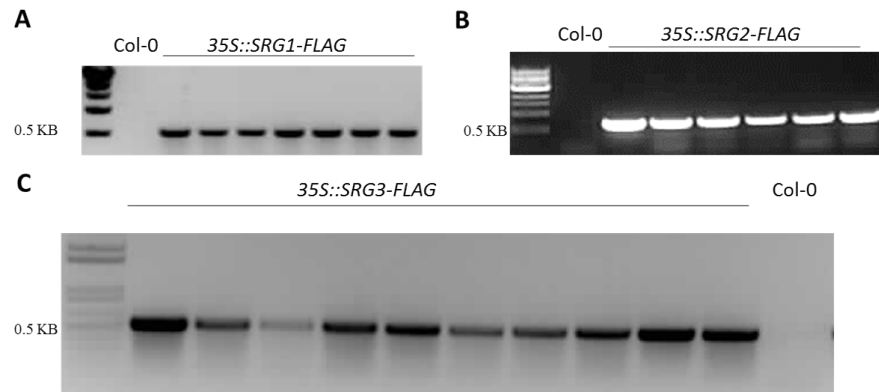
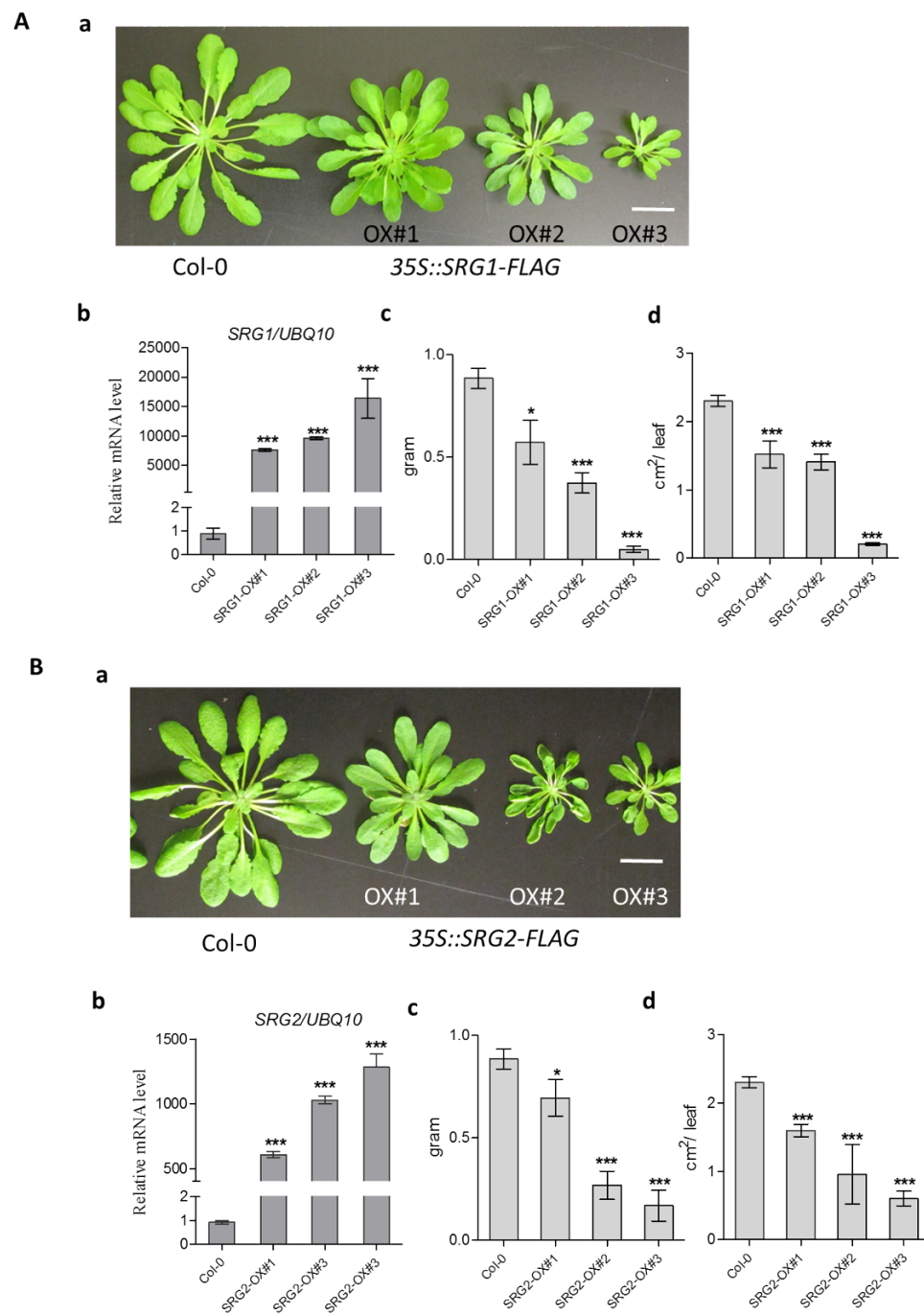


Figure 5.4 Genotyping of *SRG-OX* lines.

Sequencing primers for the pGWB11 vector were used for genotyping to confirm the transgenic plants harboured *35S::SRG1-FLAG* (A), *35S::SRG2-FLAG* (B), or *35S::SRG3-FLAG* (C). Col-0 plants were used as negative control. The genomic DNA extracted from four-week-old plants was used for PCR and the transgenic plants gave a product ~ 0.5 kb, whereas this pair of primers did not amplify any fragment from Col-0. 0.5 kb size fragments in DNA ladder are highlighted.

In order to uncover further insights into *SRG* genes function, we chose different physical sizes of *SRG-OX* lines for further testing. As we observed in figure 5.2, loss-of-function mutations in *SRG* genes enhanced *Arabidopsis* growth. On the contrary, overexpression of *SRG* genes compromised plant growth. RNA was extracted from 4-week-old plants grown under short day conditions and then qRT-PCR was conducted to test the correlation between growth and *SRG* mRNA levels. The results showed *SRG-OX* lines strongly enhanced *SRG* transcript accumulation (Figure 5.5b). Furthermore, fresh weight decreased as the *SRG* mRNA level increased in *SRG-OX* lines (Figure 5.5c). More importantly, in the *SRG3-OX* # 3 line *Arabidopsis* growth was dramatically repressed (Figure 5.5C). Additionally, we also determined the leaf area as shown in figure 5.5d and this trait also decreased as the *SRG* genes expression increased.



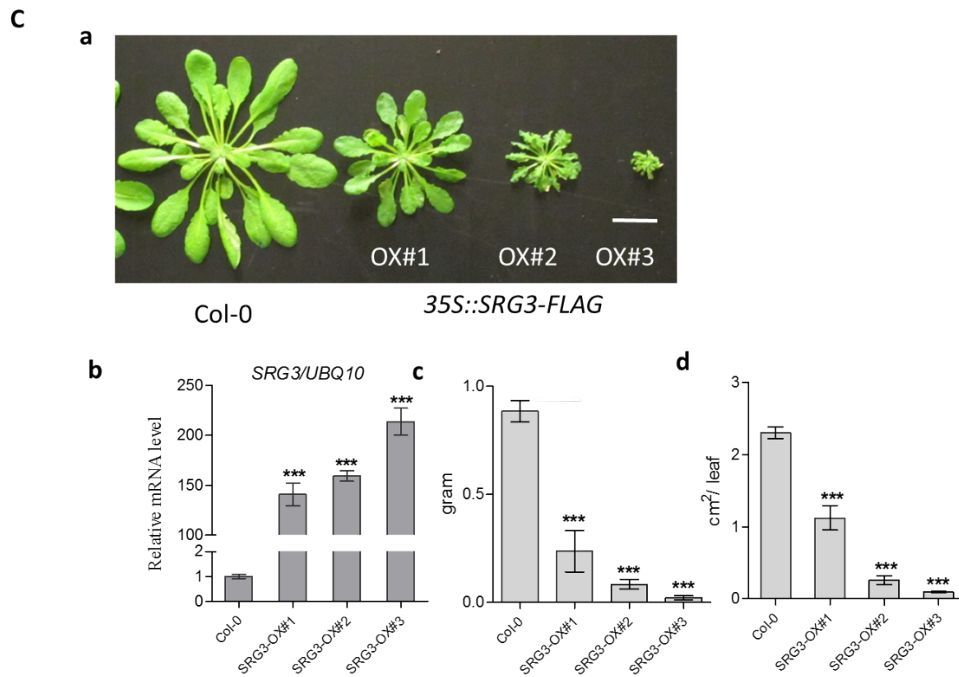


Figure 5.5 Phenotypic analysis of *SRG-OX* lines.

The phenotype of *SRG1-OX* (A), *SRG2-OX* (B) and *SRG3-OX* (C) lines were compared with Col-0. All plants were soil-grown in the same conditions as shown in figure 5.2 and the images were taken when the plants were 6-week-old. (a) All the *SRG-OX* lines showed reduced stature relative to Col-0. (b) RNA was extracted from 4-week-old *SRG-OX* lines (Indicated in a) and the transcripts of *SRG* were measured by qRT-PCR. The mRNA level of *SRG* in Col-0 was normalized as 1.0, and the mean value is derived from at least 3 independent biological replicates. (c) The fresh weight of *SRG-OX* lines and Col-0 were recorded at 6-week-old. (d) The leaf area of *SRG-OX* lines were measured and compared with Col-0. Error bars represent SD from at least 8 independent samples. Asterisk indicates the significant difference between mutant *srG* lines and Col-0 determined by student *t* test with $P < 0.05$ and triple asterisks indicate $P < 0.01$.

5.4 *SRG-OX* lines exhibit constitutive activation of defence responses

5.4.1 *SRG-OX* lines exhibit cell death

Plants have developed a system to balance between growth and immunity to achieve optimal fitness and normally constitutive immune responses result in a reduced stature phenotype ([Malinovsky et al., 2014](#), [Wang and Wang, 2014](#), [Lozano-Durán and Zipfel, 2015](#)). In our experiments, we found that reduction of *SRG*

gene expression in *srg* mutant lines enhanced *Arabidopsis* growth (Figure 5.3), while constitutive expression of *SRG* genes blunted growth (Figure 5.5). In addition, *SRG* genes were also induced by pathogen and elicitors (Chapter 3). Therefore, we hypothesized that *SRGs* may function as positive regulators of plant immunity. More importantly, the *SRG-OX* lines showed HR-like cell death symptoms (Figure 5.6A) (Dickman and Fluhr, 2013). To confirm cell death development in *SRG-OX* lines, trypan blue staining was conducted, which stains dead or dying cells (Yun et al., 2011). As shown in figure 5.6-B, more cell death developed in *SRG-OX* lines relative to Col-0. To check whether transient overexpression of *SRGs* in tobacco could induce cell death, *Agrobacterium* mediated transformation was conducted (Figure 5.6-C). The *Agrobacterium* strain GV3101 carrying *35S::SRG-GFP* was transiently introduced to tobacco (*Nicotiana benthamiana*) and cell death developed after 5 dpi. However, the nuclear localized NLS-GFP did not show much difference from background (Figure 5.6C), which also suggests overexpression of *SRG* genes lead to cell death. Trypan blue staining was also carried out to confirm the phenotype in tobacco (Figure 5.6C.b). Figure 5.6C.c showed the ratio of staining following overexpression of *SRG-GFP* genes was significantly higher than overexpression *NLS-GFP* in tobacco leaves.

Taken together, these results indicate that constitutive expression of *SRG* genes induced cell death in *Arabidopsis* and tobacco.

5.4.2 *SRG-OX* lines increased ROS

ROS are also an important early signalling response to stress, including pathogens. ROS not only mediate signalling to active defense responses but also function as antibacterial chemicals to inhibit pathogen infection. To further investigate whether *SRG-OX* lines constitutively produce ROS, ten-day-old seedlings of *srg*, Col-0 and *SRG-OX* lines were grown on 1/2 MS medium, collected and stained with 3,3'-Diaminobenzidine (DAB) solution (which stains H₂O₂ in plant tissues). Figure 5.7A.b shows increased DAB staining in *SRG1-OX* lines relative to Col-0. Thus, *SRG1-OX* lines produced more H₂O₂ compared with Col-0. Further, *SRG2-OX* and *SRG3-OX* lines showed a similar phenotype, although more H₂O₂ was observed in *SRG2-OX* lines (Figure 5.7-B.d) and *SRG3-OX* lines relative to *SRG1-OX* plants

(Figure 5.7B.f). We also tested whether the mature leaves of *SRG-OX* lines accumulated more H_2O_2 . Figure 5.7C shows that *SRG-OX* lines had significantly more staining relative to Col-0 (Figure 5.7A.b, d, e).

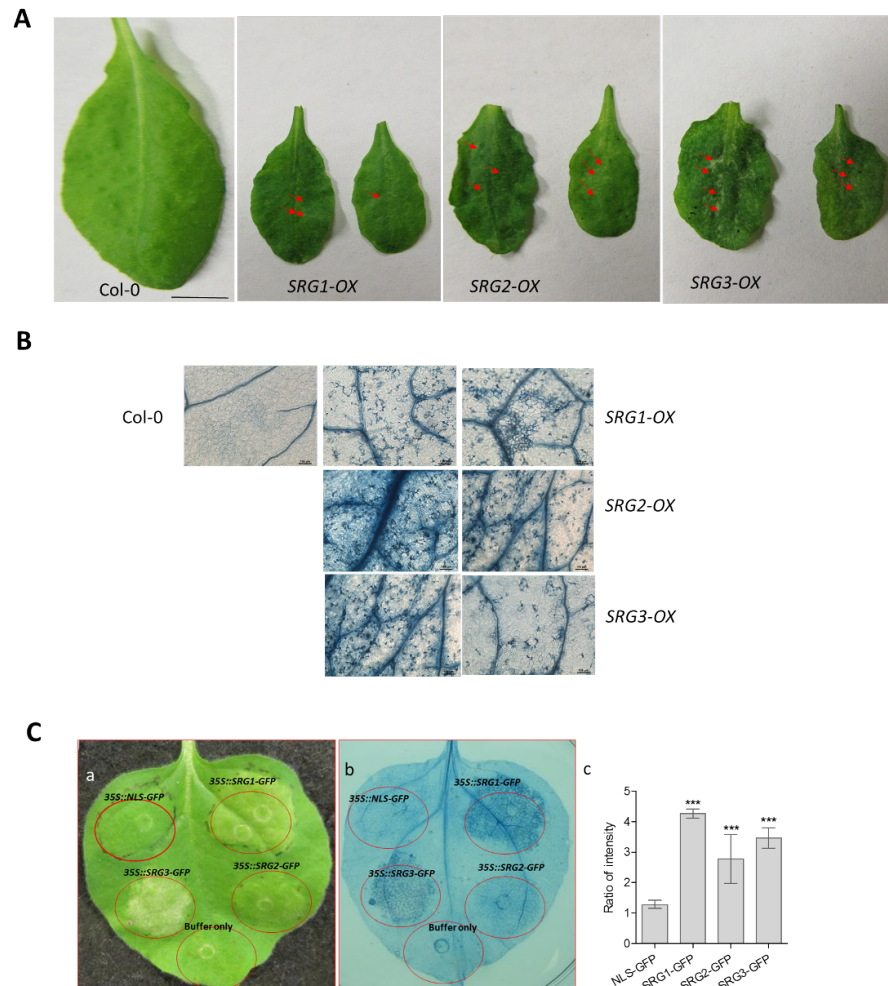


Figure 5.6 Overexpression of *SRG* genes induce cell death formation.

A, The cell death lesions in *SRG-OX* lines were highlighted by red arrows. B, Cell death development was scored by trypan blue staining and then photographs were taken under microscope. C, Transient expression of *35S::SRG1-GFP*, *35S::SRG2-GFP*, *35S::SRG3-GFP* and *35S::NLS-GFP* in tobacco was recorded after 5 dpi, (a) The visible cell death in tobacco leaf was photographed and (b) confirmation of cell death by trypan blue staining, (c) the intensity of trypan blue staining in b was quantified by image J and the NLS-GFP is normalized as 1.0. Error bars represent SD and the mean value of ratio is from at least 10 independent samples. Triple asterisk indicates the significant difference between *SRG-GFP* and *NLS-GFP* determined by student *t* test with $P < 0.01$.

Nitroblue tetrazolium (NBT) is used to analyze the level of superoxide (O_2^-), in *SRG-OX* lines and Col-0. ~10-day-old seedlings of Col-0, *SRG-OX* and *srg* lines were collected and submerged in NBT buffer for 3 hours. After destaining, the samples were photographed and analyzed. As shown in figure 5.7A *SRG1-OX* lines exhibited more NBT staining compared with Col-0, whereas the *srg1* knock out lines displayed less staining. These results suggest that the *SRG1-OX* line accumulates more O_2^- and the *srg1* mutant line produces less O_2^- . Figure 5.7A.c and figure 5.7-A.e showed *SRG2-OX* and *SRG3-OX* lines also produced more O_2^- relative to Col-0. However, *srg2* and *srg3* lines did not show any difference compared with Col-0. In order to check if the *SRG-OX* mature leaves accumulate O_2^- , leaves from 4-week-old plants under short day conditions were collected and stained with NBT solution for 3 hours. Figure 5.7-B indicates that increased staining was observed in *SRG-OX* lines, whereas Col-0 had less staining. Thus, mature leaves from *SRG-OX* plants produced more O_2^- compared with Col-0, which is consistent with seedling data (Figure 5.7A).

Histological staining with DAB and NBT showed ROS level in *SRG-OX* lines enhanced, whereas the ROS level in *srg* and Col-0 lines was at a lower level. Taken together, *SRG* genes are involved in ROS metabolism and overexpressing *SRG* induced ROS accumulation in these transgenic *Arabidopsis* plants.

5.4.3 *SRG-OX* lines increased *PR1* expression

The results above showed *SRG-OX* lines enhanced ROS production and cell death development, which suggests that *SRG-OX* lines may initiate the defence response. In order to explore this hypothesis, we chose the defence marker gene *PR1* and tested its expression in these *SRG-OX* lines. The leaves from four-week-old Col-0 and *SRG-OX* lines under short day conditions were collected and RNA was extracted for analysis. A qRT-PCR experiment was conducted. As shown in figure 5.8A, the *PR1* expression in *SRG1-OX* lines was significantly increased compared with Col-0, indicating the engagement of SA-dependent gene expression. Further, overexpressing of *SRG2* (Figure 5.8B) or *SRG3* (Figure 5.8C) also enhanced *PR1* expression. These results demonstrate that overexpression of *SRG* genes enhanced defence marker gene *PR1* expression.

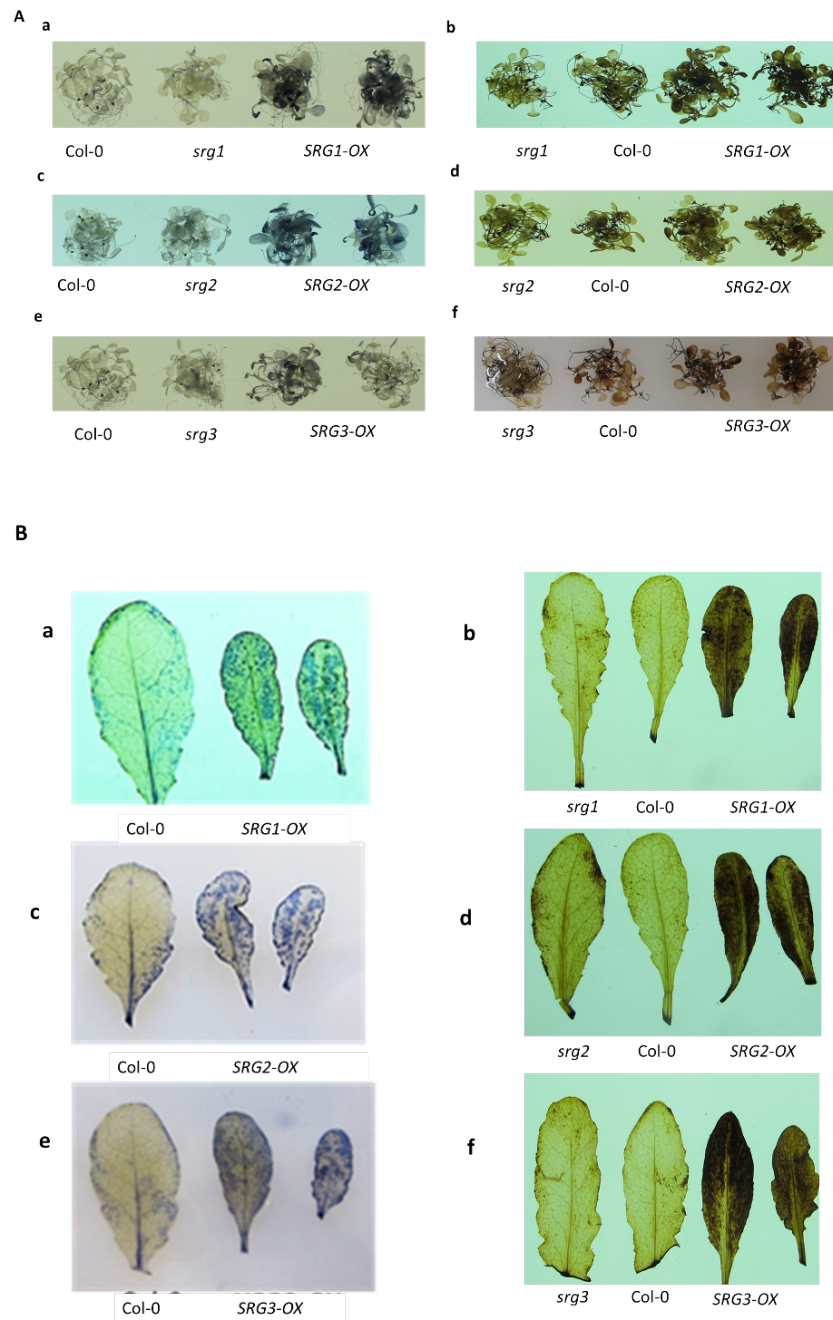


Figure 5.7 *SRG-OX* lines show enhanced ROS production relative to Col-0.

A, NBT staining (a,c and e) which reports superoxide accumulation and DAB staining (b, d and f) which monitors hydrogen peroxide accrual in seedlings of given genotypes. B, ROS level in mature leaves of given genotypes were taken after staining with NBT (a, c and e) and DAB staining (b, d and f).

Collectively, our results show overexpression of *SRG* genes activate immune responses including cell death development, ROS production and *PR1* expression.

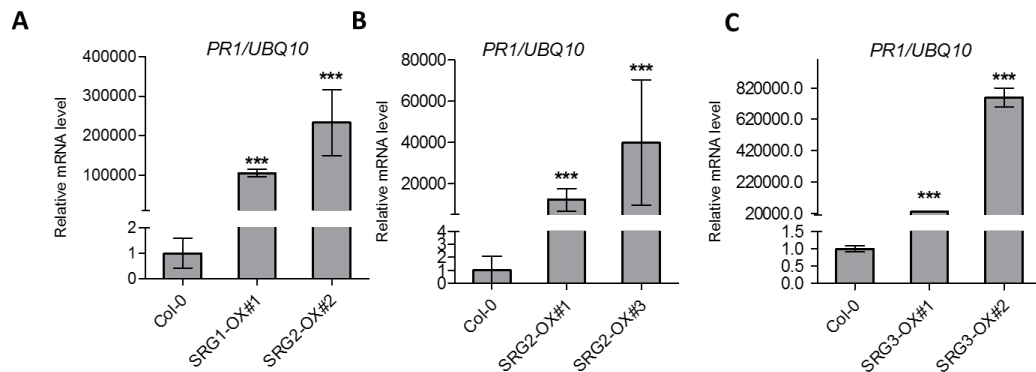


Figure 5.8 Constitutive expression of *SRG* genes enhances *PR1* expression.

RNA was extracted from 4-week-old *SRG-OX* lines and Col-0, and the expression level of *PR1* in *SRG1-OX* (A), *SRG2-OX* (B), *SRG3-OX* (C) and Col-0 is determined by qRT-PCR. *UBQ10* is used as the internal control and the mRNA level of *SRG* in Col-0 is normalized to 1.0. Error bars represent SD and the mean value is from at least 3 independent biological replicates. Triple asterisks indicate the significant difference between *SRG-OX* lines and Col-0 determined by student *t* test with $P < 0.001$.

5.5 EAR domain at C-terminus of *SRG1* and *SRG3* is required for cell death in tobacco

Overexpression of EAR-containing proteins in plants were identified to induce cell death and EAR-motif in these proteins was required for cell death development (Kazan, 2006, Lu et al., 2011, Ogata et al., 2013). For this prospect, we wanted to ask whether EAR-motif in *SRG1* and *SRG3* is essential for cell death development. In order to examine this hypothesis, the C-terminus containing the EAR motif in *SRG1/SRG3* was truncated and tested the effect of C-terminus on cell death development. Firstly, we deleted the EAR motif at C-terminus ($35S::SRG1^{\Delta}$ -GFP, $SRG1^{\Delta}$ -GFP) and checked whether this influences the localization of $SRG1^{\Delta}$ -GFP. Previous section (Figure 3.10), *SRG*-GFP was shown to localize at the nucleus. Figure 5.9A shows that $SRG1^{\Delta}$ -GFP still localized to the nucleus. This is consistent with the prediction that the nuclear localization signal in *SRG1* is located in the

middle of the protein.

Cell death development was also tested in tobacco leaves using the *Agrobacterium*-infiltration method. Cell death lesions were visible when *35S::SRG1-GFP* or *35S::SRG3-GFP* were transiently expressed, whereas *35S::SRG1^Δ-GFP* and *35S::SRG3^Δ-GFP* did not drive cell death formation (Figure 5.9B.a). In order to confirm the cell death development, trypan blue staining was carried out. Increased staining was observed when *35S::SRG1-GFP* or *35S::SRG3-GFP* were expressed (Figure 5.9B.b). In contrast, overexpression of *35S::SRG1^Δ-GFP* (*SRG1^Δ-GFP*), or *35S::SRG3^Δ-GFP* (*SRG3^Δ-GFP*) did not result in cell death formation. Further, trypan blue staining confirmed the results that truncation of the EAR domain (*SRG^Δ-GFP*) leads to loss of the ability to trigger cell death. Therefore, these findings strongly imply that the putative repressor motif EAR, located at the C-terminus in *SRG1* and *SRG3*, is essential for cell death development in transient expression in tobacco leaves.

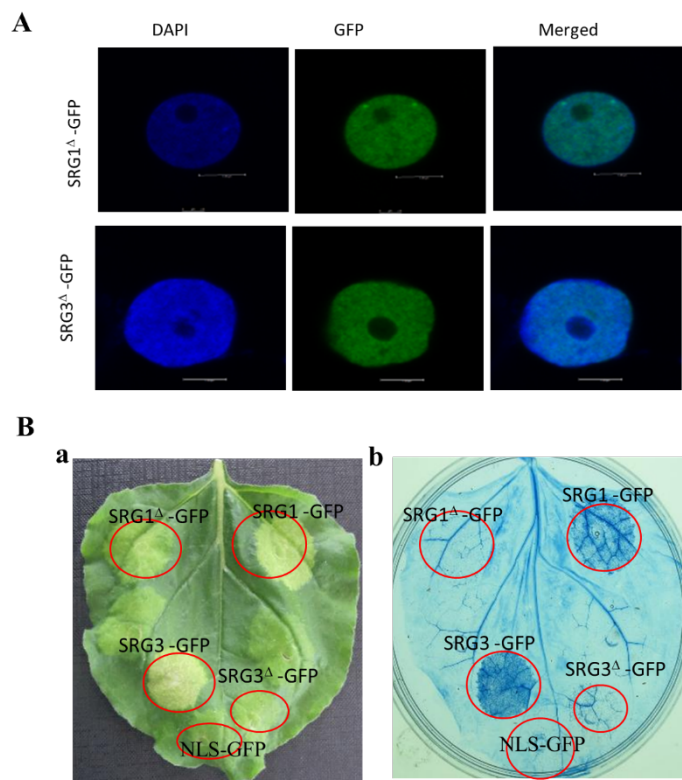


Figure 5.9 Truncation of C-terminus with EAR-motif in *SRG1* and *SRG3* blocks

cell death formation but does not impact cell localization.

A, Cellular localization of *SRG1*^Δ-GFP and *SRG3*^Δ-GFP are determined by confocal after 36 hour post transformation of *SRG*-GFP or *SRG*^Δ-GFP. DAPI staining is used for confirmation of nucleus. B, Visible cell death development of transiently expressed *SRG* genes in tobacco after 5 dpi is recorded by photograph (a) and conformation of cell death is assayed by trypan blue staining.

5.6 *SRGs* regulate plant basal defence and *R*-gene mediated resistance

SRG genes are up-regulated by *Pst* DC3000 (Figure 3.1 and 3.8) and overexpression of these *SRG* genes activates plant immune responses, including ROS production (Figure 5.7), HR-like cell death development (Figure 5.6) and *PR1* expression (Figure 5.8). These data suggests that *SRG* genes may positively regulate plant immunity. To investigate the hypothesis that *SRGs* may be positive regulators of plant immunity, the model bacterial pathogen *Pst* DC3000 was used to challenge *srg1* plants. After infiltrating leaves of given genotypes with *Pst* DC3000, the bacterial titre was recorded over time following infection. As shown in figure 5.10, the amount of infiltrated bacteria in *srg1* and Col-0 was not different at 0 dpi, which suggests that *srg1* plants can be infiltrated to similar levels as Col-0. However, the titre of *Pst* DC3000 in *srg1* was significant higher than that in Col-0 after 3 dpi (Figure 5.10A), indicating disruption of *SRG1* leads to susceptibility to *Pst* DC3000. On the contrary, less *Pst* DC3000 was detected in *SRG1-OX* lines compared with Col-0 (Figure 5.10A). Moreover, *SRG1-OX* #2, which has higher transcript levels of *SRG1* than *SRG1-OX* #1, displayed less *Pst* DC3000 growth, indicating the *SRG1* expression level is directly related to the extent of resistance to *Pst* DC3000. Taken together, these results suggest *SRG1* acts as a positive regulator of plant disease resistance against *Pst* DC3000.

In addition, we also tested the function of *SRG2* and *SRG3* in basal resistance. As shown in figure 5.10C and figure 5.10E, disruption of *SRG2* or *SRG3* mutant lines exhibited pathogen susceptibility, but constitutive expression of *SRG2* or *SRG3* enhanced resistance to *Pst* DC3000. These results suggest transcription factor *SRGs*

positively regulate plant basal resistance.

We next examined the impact of *SRG* genes on *R*-gene mediated disease resistance in *Arabidopsis*. Figure 5.10B showed that the titre of *Pst* DC3000/*avrRpm1* in *SRG1* knock-out line *srg1* was 10-fold more than that in Col-0, indicating disruption of *SRG1* lead to increased susceptibility to *Pst* DC3000/*avrRpm1*.

We also tested the effects of *SRG2* and *SRG3* in the *R*-gene mediated resistance with the same avirulence pathogen *Pst* DC3000/*avrRpm1*. Figure 5.10D and figure 5.10F showed that in *srg2* or *srg3* lines bacterial growth was promoted compared with Col-0. Collectively, these data demonstrate that *SRGs* are required for *R*-gene mediated disease resistance. Taken together, our data suggests that transcription factor *SRGs* are involved in the plant immune response.

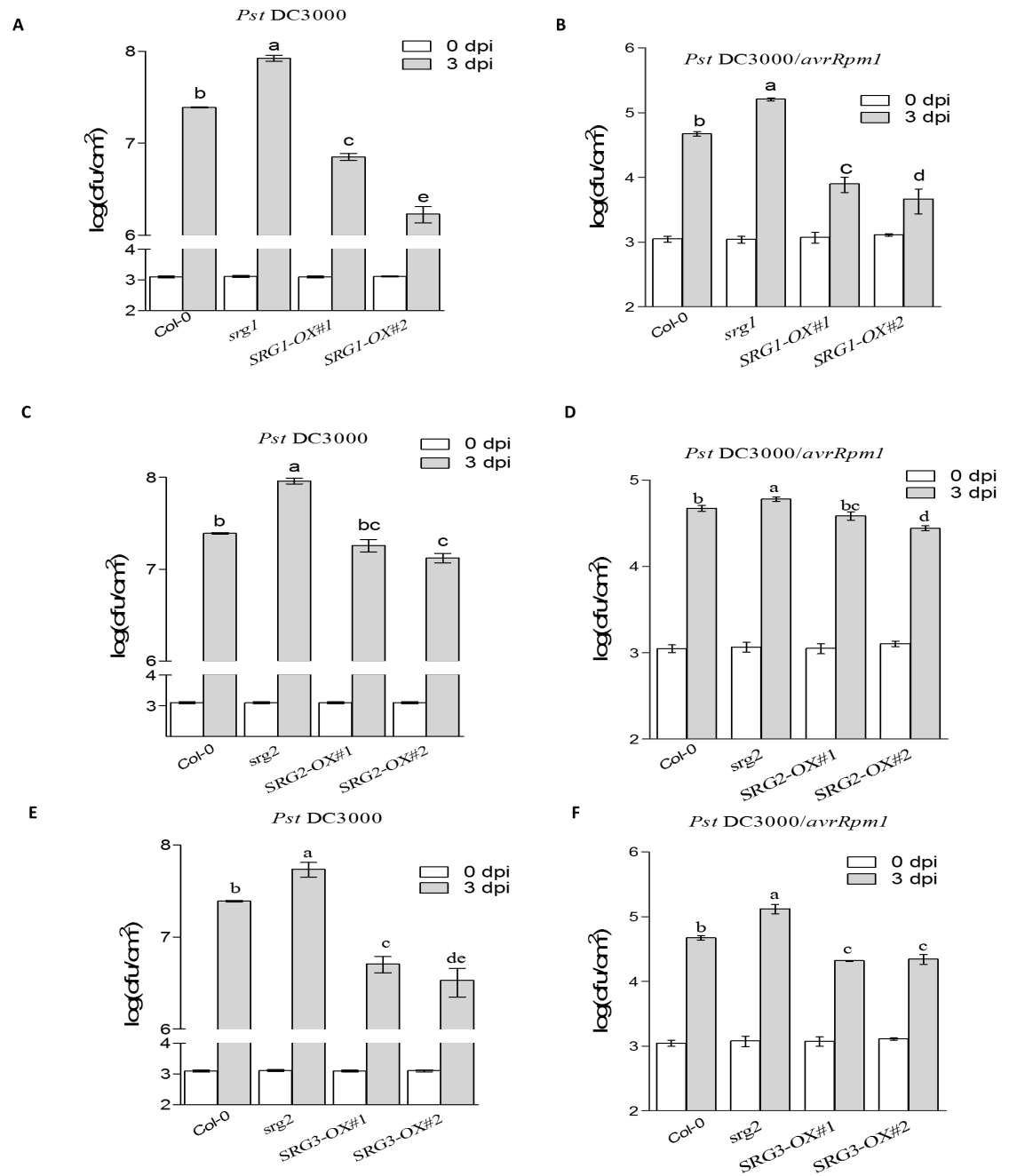


Figure 5.10 *SRG* genes are required for disease resistance.

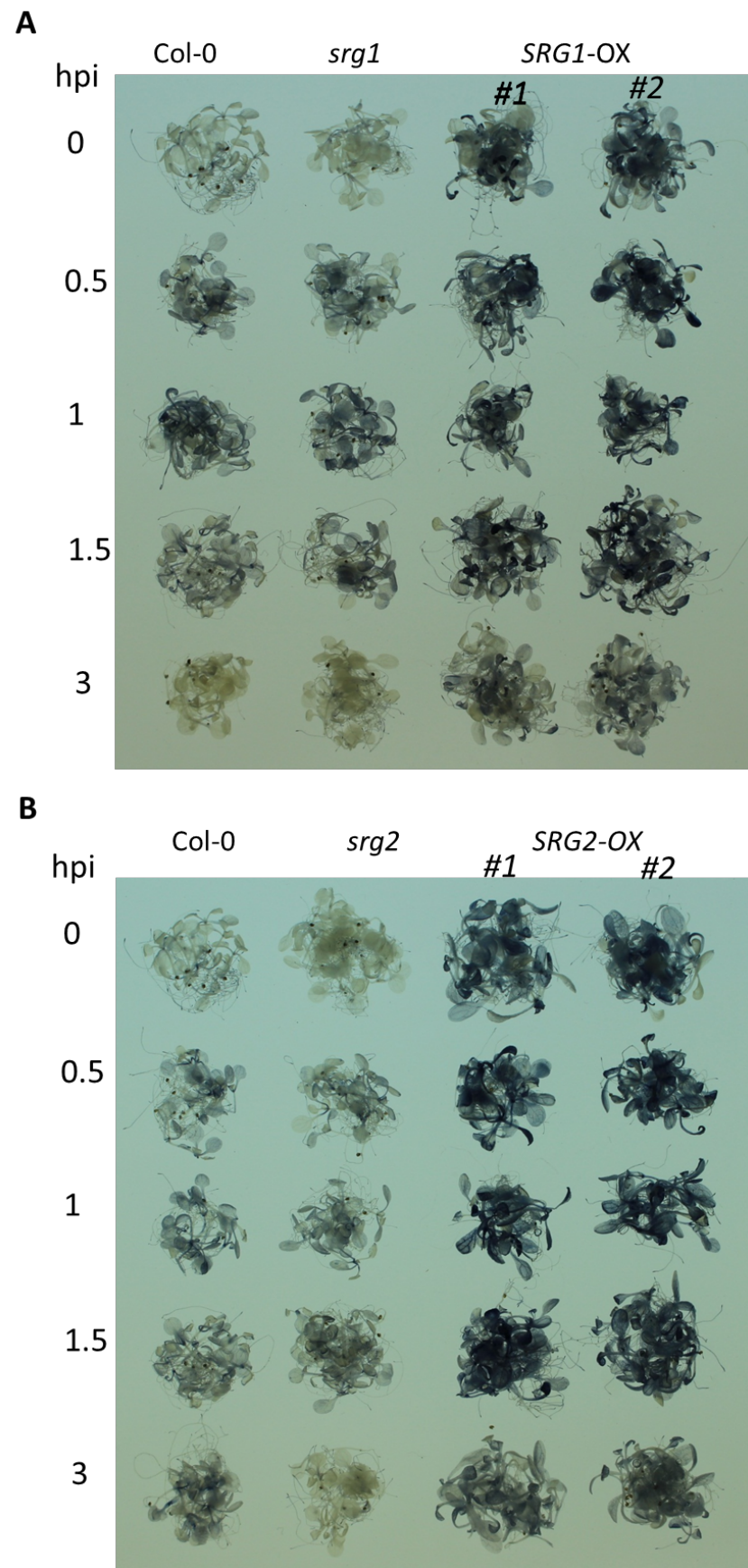
Col-0, *srg* mutants and *SRG-OX* lines were infected with *Pst* DC3000 (A, C, E) or *Pst* DC3000 carrying *avrRpm1* (B, D, F) and the growth of *Pst* DC3000 was recorded after 0 or 3 day post inoculation (dpi). Error bars represent SD and the mean value is from at least 3 independent biological replicates. Different letters indicate the significant difference between *SRG-OX* lines and Col-0 determined by student *t* test with $P < 0.05$.

5.7 *SRG* genes regulate plant defence by modulating ROS production in response to *Pst* DC3000

As shown in figure 5.7, transgenic plants that overexpressed *SRG* genes enhance ROS production. It is possible that *SRG* genes could mediate ROS production. In order to examine this hypothesis, ~10 seedlings of given genotypes were challenged with *Pst* DC3000 ($OD_{600}=0.08$), samples were collected at the indicated time points and stained with NBT solution. As shown in figure 5.11A, the NBT staining in *srg1* mutants was less than that in Col-0, and *SRG1-OX* had more staining than Col-0 at 0 hpi, which is in agreement with the data in figure 5.7. Following *Pst* DC3000 infection, the NBT staining in Col-0 and *SRG1-OX* lines increased at 0.5 hpi, whereas *srg1* lines had less staining relative to Col-0. Interestingly, $O_2^{\cdot-}$ level peaked at 1 hpi in all genotypes and the NBT staining in *srg1* and Col-0 reached to almost the same level. These results reveal that *srg1* mutant lines delay $O_2^{\cdot-}$ production in response to *Pst* C3000.

Next we tested if *SRG2* and *SRG3* have the same response. As shown in figure 5.11, the level of $O_2^{\cdot-}$ in *SRG2* (Figure 5.11-B) and *SRG3* (Figure 5.11-C) mutant lines exhibited the similar pattern in response to *Pst* DC3000. Specifically, the $O_2^{\cdot-}$ increased slowly in both *srg2* and in *srg3* mutants compared with Col-0 at 0.5 hpi, but at 3 hpi the ROS level decreased to basic level.

Taken together, these data suggests that *SRG* genes could mediate $O_2^{\cdot-}$ production in response to *Pst* DC3000 at the early phase.



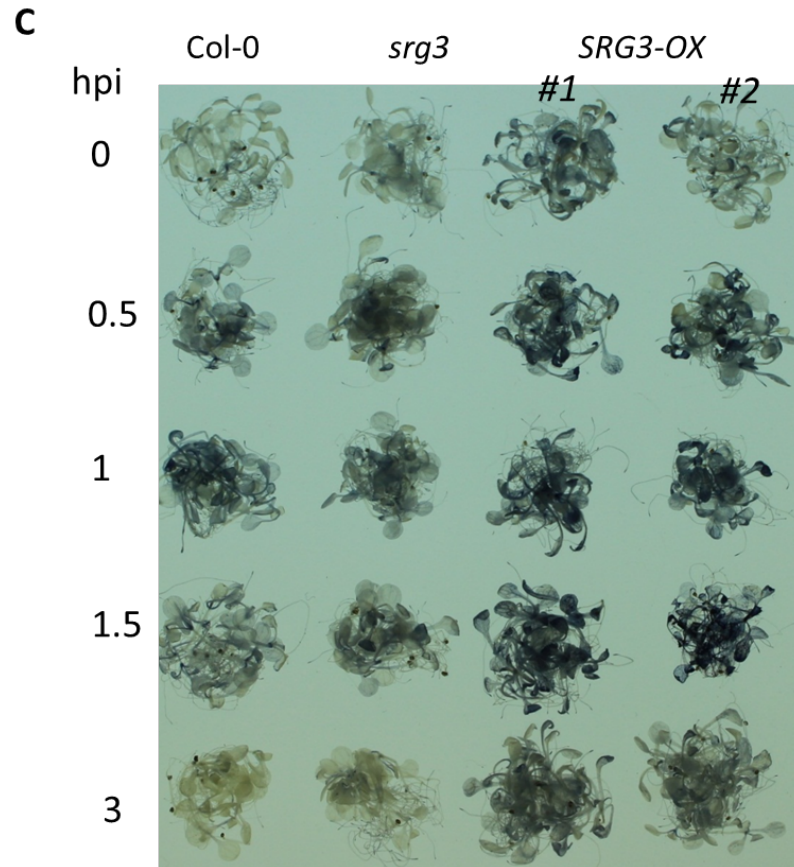


Figure 5.11 *SRG* genes regulate ROS production during pathogen infection.

~10-day-old seedlings of Col-0, *srg* mutants and *SRG-OX* lines were inoculated with *Pst* DC3000 ($OD_{600}=0.08$) and the samples were collected in the indicated time for NBT staining. After destaining the samples were photographed. hpi, hour post inoculation.

5.8 *SRG1* regulates the immune response by modulating cell death

HR plays important roles in effector-trigger immunity and we found *SRG-OX* lines triggered HR-like cell death development in plants. Moreover, *SRG1* T-DNA insertion lines *srg1* showed pathogen susceptibility and *SRG1-OX* lines enhanced disease resistance to *Pst* DC3000/*avrRpm1*, compared with Col-0. These results promoted us to ask whether *SRG1* could modulate cell death to regulate immune response. To gain further insight into the effects of *SRG1* on *R*-gene mediated immune response, leaves of *srg1*, Col-0 and *SRG1-OX* lines were infiltrated with avirulent pathogen *Pst* DC3000/*avrRpm1*. As shown in figure 5.12, after 1 dpi Col-0

and *SRG1-OX* leaves showed HR symptoms, wilting phenotype. On the contrary, the *srg1* did not show any symptoms at 1 dpi. Trypan blue staining was carried out to check HR development. As shown in figure 5.12 in response to *Pst* DC3000/*avrRpm1*, HR was developed in Col-0 and *SRG1-OX* leaves but not in *srg1* leaves at 1 dpi, but HR developed in *srg1* at 2 dpi, which suggests that *srg1* mutants delayed HR development in response to *Pst* DC3000/*avrRpm1*.

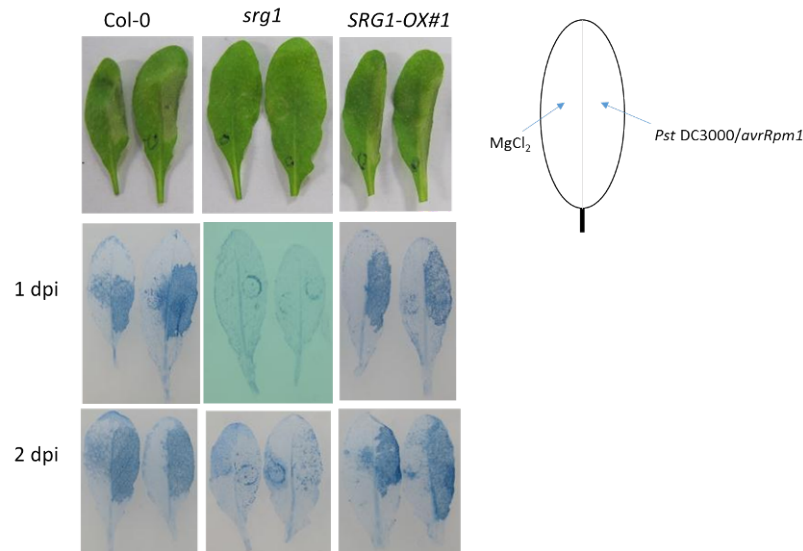


Figure 5.12 *SRG1* mediated cell death development triggered by *Pst* DC3000 carrying *avrRpm1*.

Leaves of given genotypes were injected by bacterial pathogen *Pst* DC3000 carrying *avrRpm1* in the right and MgCl_2 in the left (Shown in right picture), then the visible HR response in leaves were photographed after 2 days post inoculation (dpi) as shown in upper row. The HR development in inoculated leaves was confirmed by trypan blue staining after 1 dpi (Middle row) or 2 dpi (Bottom row). The bacterial pathogen *Pst* DC3000 carrying *avrRpm1* was suspended in 10 mM MgCl_2 (with $\text{OD}_{600}=0.02$) was used for treatment and 10 mM MgCl_2 was used for control

5.9 Discussion

Transcription factors play pivotal roles in plant immunity by reprogramming gene expression and NO is proposed to regulate the properties of transcription factors through a post translation modification, S-nitrosylation ([Durner and Klessig, 1999](#), [Hausladen and Stamler, 1998](#), [Yu et al., 2014](#)). However, little is known about how NO modulates gene expression in plant immunity. In this study, we identified NO-regulated genes *SRGs* which function in plant immunity. Moreover, we found *SRG1* and *SRG3* could also be modified by NO and this modification could blunt the DNA bind activity of *SRG1* *in vitro*. Thus, indicating *SRGs* may act as key downstream, NO-controlled, regulatory proteins in plant immunity.

Here, we verified that *SRGs* function as important regulators of both plant immunity and growth, as *SRG-OX* lines inhibited *Arabidopsis* growth (Figure 5.5) and showed disease resistance (Figure 5.10). In contrast, *srg* mutant lines compromised immune responses and enhanced *Arabidopsis* growth. Our results, together with others, suggest that the relationship between growth and immunity is antagonistic ([Fan et al., 2014](#), [Wang and Wang, 2014](#)). For example, application of elf18, a PAMP, inhibited *Arabidopsis* seedling growth, which provided direct evidence that triggering the immune response results in growth inhibition ([Malinovsky et al., 2014](#)). Genetic evidence also supports this hypothesis. Thus, *DEL1*, an atypical E2F transcriptional repressor which is required for cell proliferation, was verified to regulate the plant immune response and a *DEL1*-deficient line, *dell*, displayed blunted growth whilst exhibiting resistance to powdery mildew, *Golovinomyces orontii* ([Chandran et al., 2014](#)). Also, a basic Helix-loop-Helix (bHLH) transcription factor bHLH84, identified by forward genetic screening, mediated inhibition of *Arabidopsis* growth and resistance to pathogens when overexpressed in *Arabidopsis* ([Xu et al., 2014](#)). Therefore, our results, together with these findings, suggest that the balance between growth and immunity is complex and is becoming an important theme.

The ROS burst occurs within minutes in response to stress stimuli and increasing evidence has shown that ROS play crucial roles in immunity ([Miller et al., 2009](#), [Yoshioka et al., 2009](#), [Grant, 2000 #969](#)). Previously, *SRG2* and *SRG3* were

demonstrated to be involved in ROS signalling and their transcripts could be induced by various stimuli, such as light, salt and wounding. Our findings also showed *SRG-OX* lines enhanced ROS production (Figure 5.7), which are in agreement with reported data ([Rizhsky et al., 2004](#), [Ciftci-Yilmaz et al., 2007](#)). But the roles of *SRGs* in ROS signalling appear very complex. Interestingly, *SRGs* seems able to regulate ROS level in plants, because knockdown of *SRG2* and *SRG3* represses the expression of *ascorbate peroxidase* (*APX*), which encodes a ROS scavenging enzyme ([Ciftci-Yilmaz et al., 2007](#)). Additionally, transcriptional profiling of *SRG3-OX* lines in response to H₂O₂ showed antioxidative genes were elevated, indicating *SRGs* may be important regulators of plant ROS signalling by remodeling transcriptional expression ([Rizhsky et al., 2004](#)). However, the mechanisms underpinning *SRG* function remain to be established.

Cell death is considered as a crucial strategy against attempted pathogen infection. Also, ROS acts a signalling molecule to activate defence pathways and a high level of ROS can drive cell death, which is a strategy to inhibit biotrophic pathogen proliferation ([Gough and Cotter, 2011](#), [Zhang et al., 2015](#)). Further, RBOHD, which is the most important enzyme for ROS synthesis in plants, was reported to be S-nitrosylated and impaired its ROS accumulation, limiting cell death development ([Yun et al., 2011](#)). In our study, we found that overexpression of *SRGs* drive ROS synthesis and cell death formation, which results in the resistant phenotypes of *SRG-OX* lines to *Pst* DC3000 (Figure 5.10). Overexpression of other plant transcription factors has resulted in similar phenotypes to *SRG-OX* lines. For example, transient expression of tobacco ethylene response factor, *NtERF3*, promoted cell death development and *PRI* expression ([Ogata et al., 2013](#)). Similarly, overexpression of an *Arabidopsis* EAR-containing transcription factor, *DEAR1*, induced cell death and enhanced disease resistance to *Pst* DC3000 ([Tsutsui et al., 2009](#)). This may due to overexpression of these repressors activate defence response and repress plant growth. These results suggest that overexpression of plant transcription factors may drive the enhancement of cell death formation to mediate immune response.

In conclusion, *SRGs* regulate *Arabidopsis* growth and overexpression of *SRG*

genes enhanced ROS production, *PR1* expression and cell death development. Furthermore, our data suggests *SRGs* positively regulate the plant defence response and we conclude that *SRGs* play important roles in plant immunity.

Chapter

6 S-nitrosylation of SRG1 negatively regulates its repressor function in plant immunity

6.1 Introduction

In response to environmental stimuli, plants have evolved various systems to perceive and transmit the stress signalling into plant cells, resulting in activation of corresponding pathway against the stress. During these physiological processes, plant transcription factors play pivotal roles in the stress response by modulating signal transduction ([Singh et al., 2002](#), [Baena-Gonzalez et al., 2007](#)). WRKY transcription factors, one of the largest families of transcriptional regulators in plants are thought to play a key role in this process ([Eulgem and Somssich, 2007](#), [Pandey and Somssich, 2009](#)). Therefore, the transcriptional activity of transcription factors involved in plant stress needs to be controlled in both time and space. Posttranslational modification, such as phosphorylation and ubiquitination, are an important mechanism to control transcriptional activity ([Spoel et al., 2010](#)). Additionally, S-nitrosylation, a redox-dependent modification by the addition of an NO moiety to a Cys thiol to form an S-nitrosothiol, is emerging as an modification for regulation of transcriptional activity ([Wang et al., 2006](#), [Yu et al., 2012](#)). For example, TGA1 ([Lindermayr et al., 2010](#)) and AtMYB30 ([Tavares et al., 2014](#)), which play important functions in plants, can be regulated by S-nitrosylation *in vitro*, but exactly how NO regulates these two transcription factors *in vivo* remains unclear. Further, NO has been shown to modify some key regulators of plant stress responses and development by S-nitrosylation ([Yu et al., 2012](#)). Transcriptome analysis suggests that NO could reprogram gene expression ([Palmieri et al., 2008](#), [Ahlfors et al., 2009](#)). However, little is known about the mechanisms of NO in association with gene expression. In order to understand how NO reprograms transcription, we identified a small group of zinc finger containing transcription factors, SRGs that could be regulated by NO. Moreover, DNA binding activity of SRG1 could be modified by NO *in vitro*, which implies that SRG1 may be an important NO regulator. Therefore, in this chapter, we will try to uncover whether NO governs SRG1 activity to aid the reprogramming of transcription

in plant immunity.

6.1 SRG1 could be S-nitrosylated *in vivo*

As shown in chapter 4, SRG1 could be S-nitrosylated by NO *in vitro* and its DNA binding activity was diminished in the presence of NO, which suggests that S-nitrosylation of SRG1 results in impairment of its transcriptional activity. We therefore tested whether S-nitrosylation of SRG1 occurs *in vivo*. Firstly, we tried to express 35S::*SRG1-FLAG* in *Arabidopsis* protoplasts using PEG-mediated transformation (Yoo et al., 2007). The extraction of total proteins from the protoplasts with or without transforming 35S::*SRG1-FLAG* was subjected to western blotting against FLAG for confirmation of SRG1-FLAG in plants. As shown in figure 6.1-A, SRG1-FLAG could be detected, whereas there is no signal in the Col-0 control. In order to determine the formation of SRG1-SNO *in vivo*, total proteins were extracted after treatment with 1 mM GSNO for 10 minutes. The total proteins were subjected to biotin-switch assay and then S-nitrosylated proteins were purified by streptavidin-agarose. The purified proteins were subjected to western blotting against FLAG. Figure 6.1B showed that SRG1 could be S-nitrosylated by GSNO *in vivo*.

We next determined whether S-nitrosylation of SRG1 occurred in the *gsnor1-3* mutant, which exhibits a high SNO level relative to wild-type. A biotin-switch assay showed SRG1-SNO formation was detected in *gsnor1-3* protoplasts by transiently expression of 35S::*SRG1-FLAG* (Figure 6.1C), but not in the Col-0 control, implying that SRG1 could be S-nitrosylated in a *gsnor1-3* background.. Collectively, SRG1 could be S-nitrosylated *in vivo* and this modification was increased in a *gsnor1-3* mutant line.

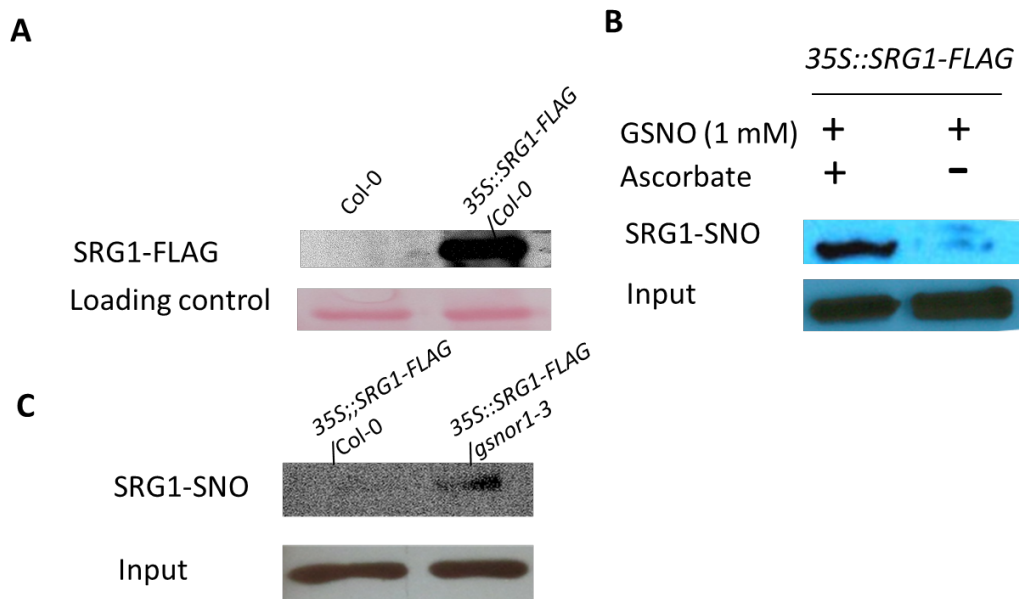


Figure 6.1 S-nitrosylation of SRG1 *in vivo*.

(a) *In vivo* expression of SRG1 using *35S::SRG1-FLAG* constructs in the Col-0 background. The extraction of total proteins from Col-0 protoplasts with or without expressing *35S::SRG1-FLAG* construct was subjected to western blotting against anti-FLAG antibody. Loading control was presented by ponceau staining to ensure the equal loading. (b) S-nitrosylation of SRG1 by GSNO *in vivo*. The Col-0 protoplasts expressed *35S::SRG1-FLAG* were exposed to GSNO and then the total proteins were collected and subjected to biotin-switch assay. Without ascorbate was served as the negative control. (C) The total protein from the Col-0 and *gsnor1-3* protoplasts expressing a *35S::SRG1-FLAG* construct were subjected to a biotin-switch assay for determining the formation of SRG1-SNO in given genotypes. Input presents the total loading of SRG1-FLAG confirmed by FLAG antibody.

6.2 S-nitrosylation of SRG1 inhibits its repression activity

A previous transcriptional repression activity assay demonstrated that SRG1 functions as a repressor. Together with the results that S-nitrosylation of SRG1 affects its DNA binding activity, it is highly possible that NO could regulate its repression activity through the redox-dependent PTM, S-nitrosylation. Based this hypothesis, we tested whether the transcriptional repression activity of SRG1 is sensitive to NO. As shown in figure 6.2A, compared with the control, the relative luciferase activity of GAL4-DB-SRG1 increased in the presence of the NO donor, SNP, indicating that NO

could inhibit SRG1 repression activity. Further, we next assayed whether SRG1 repression activity was inhibited in a *gsnor1-3* mutant as SRG1-SNO formation was enhanced in *gsnor1-3* background. Figure 6.2B showed the repression activity of SRG1 was blunted in a *gsnor1-3* background as the luciferase activity of GAL4-DB-SRG1 was restored to GAL4-DB level. These results suggest that NO negatively regulates SRG1 repression activity, likely via S-nitrosylation.

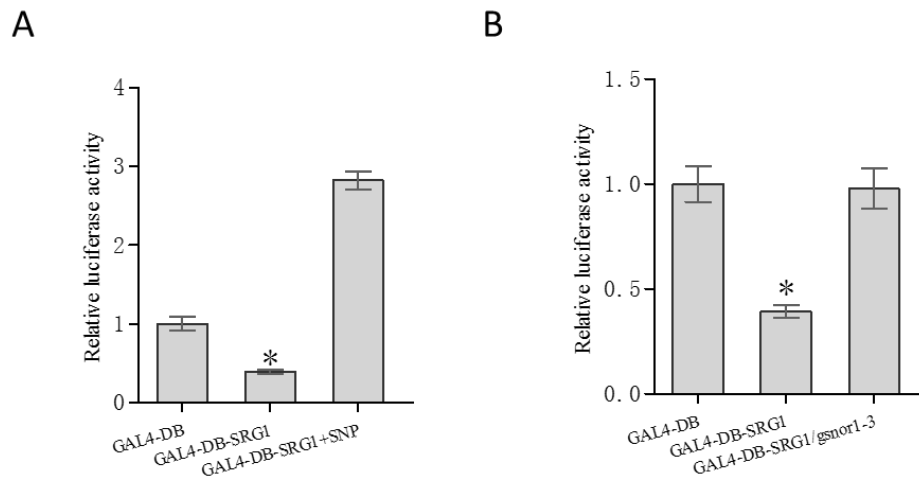


Figure 6.2 Inhibition of SRG1 transcriptional activity by NO *in vivo*.

(A) Effects of SNP on the transcriptional activity of SRG1 *in vivo*. Luciferase activity was measured following exposing the protoplasts to SNP and transient repression activity was conducted as in figure 4.14. (B) The repression activity was decreased in *gsnor1-3* plants. SRG1-FLAG was expressed in Col-0 and *gsnor1-3* protoplasts, separately, for transient repression activity assays. Luciferase activity of expressing *GAL4-DB* was normalized as 1. Error bars represent mean \pm SD and the mean value is from at least 3 independent biological replicates. Asterisks indicate the significant difference with *GAL4-DB* by student's *t* test with $P < 0.05$.

6.3 Identification and characterization of *SRG-OX gsnor1-3* plants

Because the extent of SRG1-SNO formation could be enhanced in *gsnor1-3* plants and this redox modification also appears to blunt the transcriptional activity of SRG1 *in vitro* and *in vivo*, we reasoned that S-nitrosylation of SRG1 might affect its function in plant immunity. To test this hypothesis, *SRG1-OX #1* line was crossed with *gsnor1-3* to generate *SRG1-OX gsnor1-3* plants. The genomic DNA extracted

from F2 lines was genotyped based on the three primers (LB+LP+RP) by PCR. Figure 6.3 showed that line #2 to #6 contained a T-DNA insertion homozygous at the *GSNOR1* site. Further, all selected lines had insertion for *SRG1-OX* because all these lines were screened on medium with kanamycin. The result indicated that lines #2-#6 are *SRG-OX gsnor1-3* plants and the seeds of #2 to #6 were collected for further experiments.

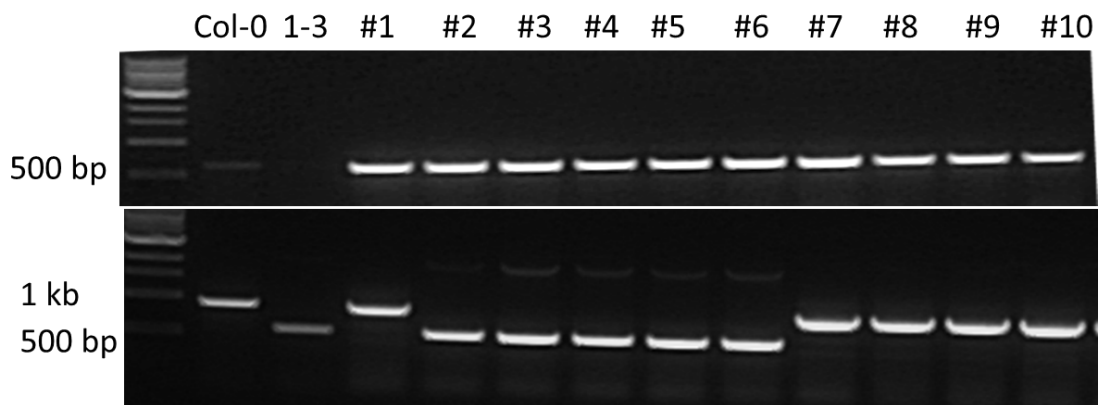


Figure 6.3 Identification of mutants *SRG-OX/gsnor1-3*.

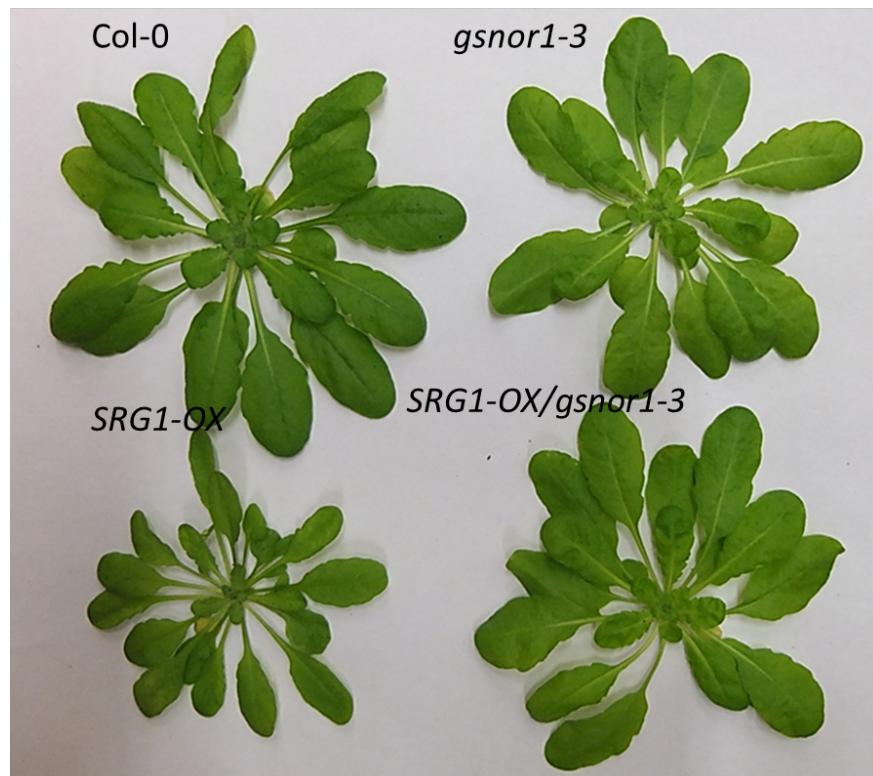
Three primers including *gsnor1-3* gene specific primers and T-DNA insertion LB board primer were employed for genotyping of *SRG-OX gsnor1-3* plants to identify homozygous *gsnor1-3* lines. *gsnor1-3* homozygous lines gave a product of ~500 bp and for Col-0 gave a product of 1 kb. Confirmation of *SRG1-OX* lines was obtained using pGWB11 vector sequencing primers, which give an expected product of ~500 bp. A DNA ladder of 500 bp and 1 kb is shown.

6.4 Phenotypic analysis of *SRG-OX gsnor1-3* plants

As *SRG1-OX* lines exhibited inhibition of *Arabidopsis* growth (Figure 5.5) and SRG1 S-nitrosylation could be increased in the *gsnor1-3* mutant (Figure 6.1), inhibiting its repression activity, we expected that formation of SRG1-SNO might affect the morphological phenotype of *SRG-OX* line. Therefore, the phenotypes of *SRG1-OX gsnor1-3* plants were investigated. Figure 6.4 shows that the *SRG1-OX* line was relatively smaller than Col-0, which is consistent with the data in figure 5.5, however, *SRG1-OX gsnor1-3* plants resembled *gsnor1-3* in terms of size, rather than the *SRG1-OX* line, which suggests that *SRG1-OX*-mediated inhibition of *Arabidopsis*

growth is repressed by high SNO levels in a *gsnor1-3* background. Therefore, we tested whether high SNO affects *SRG1* expression in these lines by qRT-PCR, as previously we have demonstrated that SRG1 can bind to its own promoter sequence suggesting it has autoregulatory activity. As shown in figure 6.4, the mRNA level of *SRG1* in *SRG1-OX gsnor1-3* plants was higher than that in the *SRG1-OX* line, which suggests NO positively increased *SRG1* expression. Thus, in combination with the EMSA data in chapter 4, it is possible that SRG1 gives negative feedback to control its own gene expression, but high SNO levels impair its repression activity, as demonstrated in a *gsnor1-3* mutant, resulting in increased *SRG1* expression. Even though transcripts of *SRG1* increased only two-fold in *SRG1-OX/gsnor1-3* plants, growth suppression was still blunted, which suggests that NO can negatively modulate *SRG1-OX* phenotypes. Collectively, these results suggest SRG1-SNO formation negatively regulates SRG1 function with respect to plant growth.

A



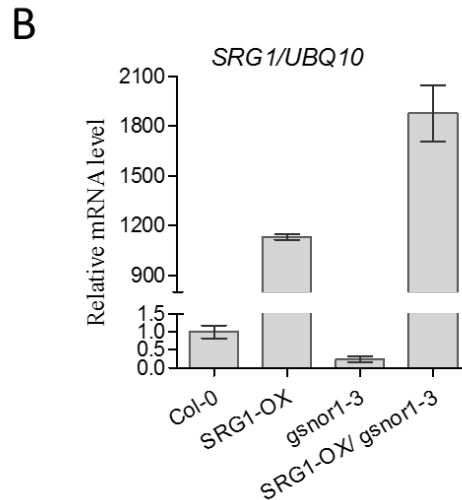


Figure 6.4 Phenotypic and molecular characterization of *SRG1-OX gsnor1-3* plants.

(A) The phenotype of 6-week-old indicated lines under short day conditions. (B) Relative mRNA level of *SRG1* in the given lines as shown in (A). The *UBQ10* gene is served as an internal control and the mRNA level of *SRG1* in Col-0 was normalized as 1. The error bars indicate the mean \pm SD from three independent biological replicates.

6.5 The repression activity of SRG1 in plant immunity is blunted in *gsnor1-3* plants

Our findings suggest that NO negatively regulates the DNA binding and by extension the transcriptional activity of SRG1. Thus, the biological consequences of S-nitrosylation of SRG1 in the immune response were investigated. We found that the constitutive resistance phenotypes of *SRG1-OX* were restored in *SRG1-OX gsnor1-3* plants. Specifically, as shown in figure 6.5 the ROS level, quantified by NBT and DAB staining, in double mutants *SRG1-OX/gsnor1-3* was recovered relative to *SRG1-OX* lines, indicating SRG1-SNO formation blunt ROS burst in *SRG1-OX* lines. Meanwhile, *PR1* expression in *SRG1-OX* was totally reduced in *SRG1-OX gsnor1-3* plants in relation to *SRG1-OX* lines (Figure 6.5). These results demonstrate that S-nitrosylation of SRG1 negatively altered its roles in immunity.

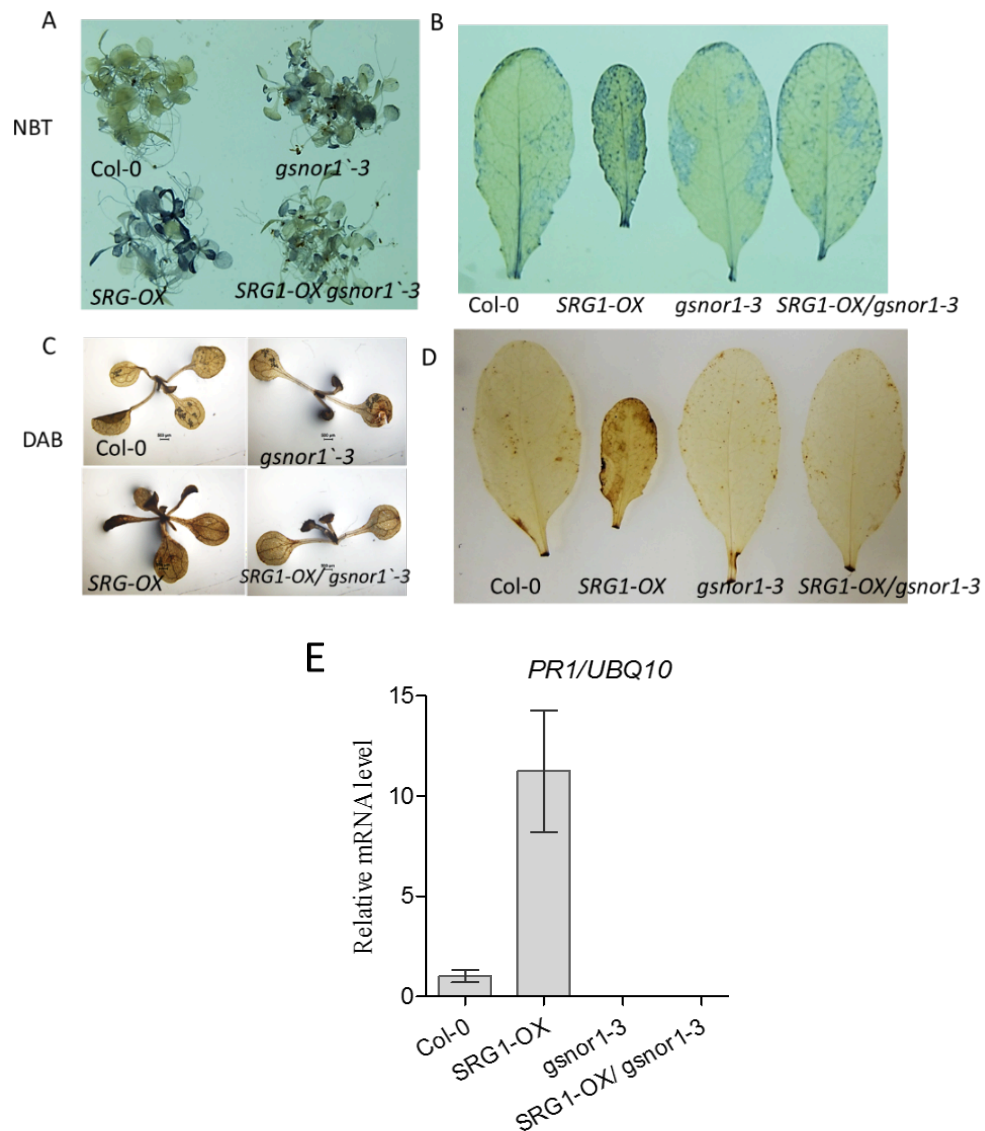


Figure 6.5 Characterization of resistance phenotype in *SRG1-OX gsnor1-3* plants.

NBT staining which reports superoxide accumulation (A, seedlings and B, mature leaves) and DAB staining (C, seedlings and D, mature leaves) which monitors hydrogen peroxide accrual in seedlings of given genotypes. E, the expression level of *PR1* in given genotypes is determined by qRT-PCR. For qRT-PCR, *UBQ10* is used as internal control and the mRNA level of *SRG* in Col-0 is normalized to 1. Error bars represent mean \pm SD and the mean value is from at least 3 independent biological replicates.

To further explore the biological consequences of this redox modification of SRG1 in plant immunity, *Pst* DC3000 was employed to test the basal immunity in

these lines, with bacterial growth recorded after 3 dpi. As shown in figure 6.6A, the titre of *Pst* DC3000 in Col-0 was higher than that in the *SRG1-OX* lines, which indicates *SRG1-OX* lines exhibit resistance, in agreement with our previous data. In contrast, the titre of *Pst* DC3000 in *SRG1-OX gsnor1-3* plants was almost the same as that in Col-0, suggesting that S-nitrosylation of SRG1 blunts its repression activity. Furthermore, we also tested whether this modification affected the *R*-gene mediated response. Thus, *Pst* DC3000/*avrRpm1* was used and the bacterial growth was recorded after 3 dpi. As expected, *SRG1-OX* plants showed increased resistance whereas the resistance in *SRG1-OX gsnor1-3* plants was restored to Col-0 levels. These data suggest that S-nitrosylation of SRG1 can blunt its role as a suppressor in plant immunity.

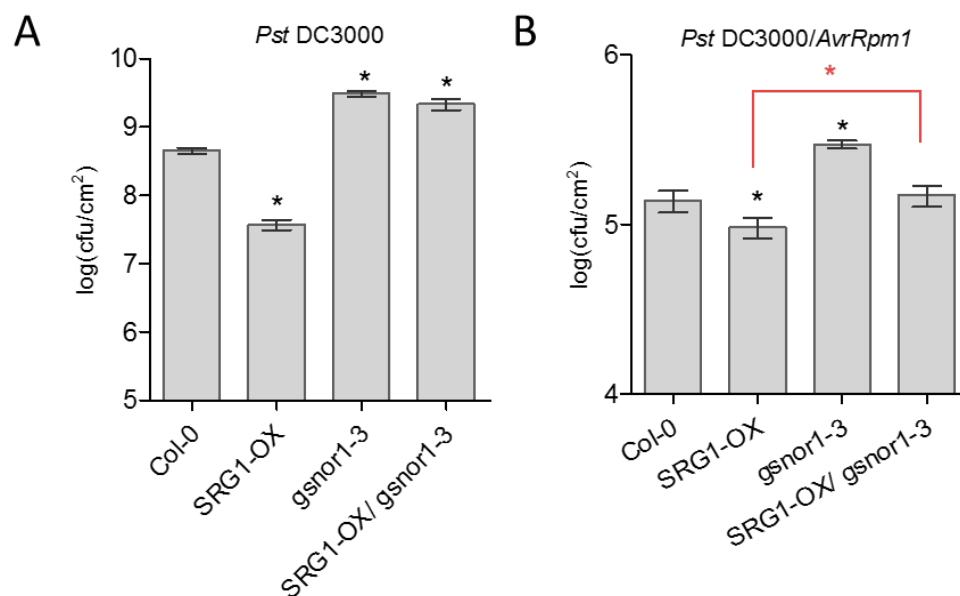


Figure 6.6 High level of SNO in *SRG1-OX* plants promotes disease susceptibility.

Col-0, *gsnor1-3* mutants, *SRG1-OX* lines and double mutants *SRG1-OX/gsnor1-3* were infected with *Pst* DC3000 (A) or *Pst* DC3000 carrying *avrRpm1* (B) and the growth of bacterial was recorded after 3 day post inoculation (dpi). Error bars represent mean \pm SD and the mean value is from 3 independent biological replicates. Star (*) indicates the significant difference between *SRG1-OX* lines and Col-0 determined by student's *t* test with $P < 0.05$.

6.6 Discussion

As an important redox molecule, NO plays important roles in plants and its activity could be transferred by S-nitrosylation ([Wang et al., 2006](#), [Yu et al., 2014](#)). Data shows NO may also modulate transcript levels in response to pathogen challenge ([Parani et al., 2004](#)). Further, NO was reported to modulate transcriptional activity of some transcription factors in eukaryotes ([Sha and Marshall, 2012](#)). For example, S-nitrosylation of human transcription factor myocyte enhancer factor 2 (MEF2) inhibited its DNA binding activity and transcriptional activity to mediate neurogenesis and cell death ([Okamoto et al., 2014](#)). Also, regulation of transcriptional activity of transcription factors by S-nitrosylation in *Arabidopsis* was identified. TGA1 ([Lindermayr et al., 2010](#)) and AtMYB30 ([Tavares et al., 2014](#)), transcriptional activity might be regulated by this redox modification *in vitro*, although the mechanisms associated with S-nitrosylation of TGA1 and atMYB30 *in vivo* have not been investigated. Cysteine residues in zinc finger transcription factors are proposed to function as NO targets in animals and it is possible that S-nitrosylation of zinc finger domains might regulate their transcriptional activity ([Kröncke, 2001](#)). In this study, we identified a SNO-regulated gene *SRG1*, which encodes a zinc finger containing transcription factor. Further, we showed that the transcriptional activity of SRG1 might be modified by S-nitrosylation. Also, we found the transcriptional activity of SRG1 was negatively regulated by NO *in vivo* (Figure 6.2). Correspondingly, the transcriptional activity of SRG1 in *gsnor1-3* plants, which have high S-nitrosothiols levels, was impaired (Figure 6.2). Remarkably, we also demonstrated that *gsnor1-3* could suppress the repression activity of SRG1. Our findings strongly imply that NO could modify transcriptional activity, at least in the case of SRG1, in *Arabidopsis* to modulate global transcription levels. However, to better understand how NO might reprogramme gene expression in plants, more SNO-regulated transcription factors would need to be identified and characterized.

Gene expression in plants is regulated precisely at the translational level and transcriptional repressors are emerging as important regulators in response to environmental stimuli ([Gutterson and Reuber, 2004](#), [Pré et al., 2008](#)). Recently, the EAR-motif containing proteins have been shown to play crucial roles in plant defence

(Ciftci-Yilmaz et al., 2007, Kagale and Rozwadowski, 2011b, Lu et al., 2011). In our study, we found SRG1, an EAR-motif containing transcription factor regulates plant immunity. Interestingly, we found overexpression of *SRG1* enhanced transcripts of *PR1* and lead to increased basal resistance. In this respect, we assumed that SRG1 possibly represses a repressor of defence-related genes, leading to the initiation of immune response gene expression. Degradation of repressors by the 26S proteasome system is a well known pathway to remove their activity (Wang et al., 2009a, Hamel et al., 2011). Our data imply SRG1 could be recognized by MAPK3/MAPK6 *in vivo* and *in vitro*, possibly resulting in phosphorylation and leading to degradation by the proteasome. The degradation of SRG1 could be blocked by MG132, an inhibitor for the 26S proteasome system, providing further evidence for our hypothesis. In addition, we found NO could blunt the transcriptional repression activity of SRG1 through S-nitrosylation. These results suggest that SRG1 might be regulated by possibly at least two parallel PTM, phosphorylation and S-nitrosylation. Therefore, it is likely that plants have evolved more than one pathway to control transcriptional activity of SRG1.

As NO increases extensively at the late stage of pathogen infection, it could initiate a negative feedback loop to inhibit SRG1 repression activity by S-nitrosylation. Genetic experiments also suggested that the resistance phenotypes of *SRG1-OX* lines were restored in high SNO levels (Figure 6.5 and figure 6.6). The balance between plant defence responses and growth is carefully regulated (Chandran et al., 2014, Wang and Wang, 2014, Lozano-Durán and Zipfel, 2015). Our finding show growth of *SRG1-OX* lines was inhibited due to a constitutive defence response (Figure 5.5). Several other *Arabidopsis* mutant lines, which exhibited constitutive resistance typically, show growth inhibition (Chandran et al., 2014, Fan et al., 2014, Malinovsky et al., 2014). However, in a *gsnor1-3* background, the growth inhibition of *SRG1-OX* lines was suppressed (Figure 6.4). Structurally, NO could induce *SRG1* expression in response to pathogen challenge and also NO negatively regulates SRG1 activity at a later stage to maintain plant fitness. Our findings provide direct evidence to support the hypothesis that NO regulates translational activity of transcription factors to modulate gene expression and govern plant immunity. In this regard, SRG1 may act as a global regulator of NO-mediated transcriptional regulation in plant

immunity.

Chapter

7 General discussion

7.1 SRGs function as positive regulators of plant immunity

With many zinc finger-type transcription factors (ZnTFs) having been identified, the abiotic stress responses mediated by zinc finger containing transcription factors in plants are relatively well understood ([Ciftci-Yilmaz and Mittler, 2008](#), [Kielbowicz-Matuk, 2012](#), [Shi et al., 2014](#)). However, the role of ZnTFs in plant immune responses are relatively unexplored. In our study, we demonstrate that *SRGs* (SNO-Regulated Gene), belonging to the C2H2-type transcription factor family, play an important role in regulating plant immunity. The observations that transcripts of *SRGs* accumulate in response to *Pst* DC3000, suggests that *SRGs* may be involved in plant immunity (Figure 3.1, 3.5, 3.6 and 3.8). Previous reports also showed that a variety of stress stimuli could elevate expression of *SRG2* (known also as *AtZAT7*) and *SRG3* (known also as *AtZAT12*) ([Rizhsky et al., 2004](#), [Ciftci-Yilmaz et al., 2007](#)). Further, stable transgenic plants that overexpressed *SRGs* enhanced resistance to *Pst* DC3000 (Figure 5.10). In contrast, T-DNA insertion mutant lines of *SRG* compromised resistance to *Pst*DC3000 (Figure 5.10). Other C2H2-type transcription factors, *AtZAT6* ([Shi et al., 2014](#)) and *AtZAT10* ([Mittler et al., 2006](#)) have also been implicated in plant biotic and abiotic stress, respectively. These data imply that C2H2-type proteins may be central positive regulators of plant stress responses, including plant immunity.

Previously, *SRG3* was proposed to play a central role in ROS-mediated plant stress signalling ([Davletova et al., 2005](#)). The data here showed that overexpression of *SRG* genes accumulated ROS and in *srg* mutants the ROS burst was delayed in response to *Pst* DC3000 also supports this hypothesis (Figure 5.11). However, the exact role of *SRGs* in ROS signalling may be very complex, because *SRGs* seem to act both downstream and upstream of ROS. On the one hand expression of *SRGs* partly results from ROS burst in response to stresses ([Miller et al., 2009](#)), but on the other the repression of *SRG* transcripts can delay ROS production in response to *Pst*

DC3000 (Figure 5.11). Similar results were observed that the expression of *SRG3* was delayed in the *RbohD* (*respiratory burst oxidase homolog D*, one of the most important NADPH oxidases enzyme in plants) mutants, which can delay ROS accumulation, in response to wounding ([Miller et al., 2009](#)). Correspondingly, the results that overexpressing *SRG3* induced *RbohD* expression strengthened this link between *SRGs* and ROS.

As a high level of ROS in plants could cause cell death development and we found that overexpression of *SRGs* can induce HR-like cell death formation (Figure 5.6), it is possible that *SRG-OX* lines induced cell death indirectly, maybe by enhancing a ROS burst. Therefore, these findings in combination with previous data imply that the proteins belonging to C2H2-type might be important regulators of ROS signalling.

To date, the EAR-motif, a putative repressor domain has been implicated as a key regulator of plant gene expression ([Kazan, 2006](#), [Dong and Liu, 2010](#)). Sequence analysis reveals that these *SRGs* contain an EAR-motif and a transient repressor assay shows that *SRGs* are active repressors (Figure 4.1 and 4.14). It is reasonable conjecture that *SRGs* regulate transcriptional activity to reprogramme the transcriptome in response to a variety of stresses. EMSA data in figure 4.7 indicates that *SRG1* can specifically bind to DNA with repeated AGT sequences. It is therefore speculated that *SRGs* may recognize target genes with repeated AGT motifs in their promoter region to repress its transcription. Notably, the EAR motif is proposed to interact with the co-repressor Topless to form a repressor complex and that subsequently functions as a transcriptional repressor. Protein interactions between *SRG1* and Topless in chapter 4 suggest that *SRGs* might recruit the co-repressor Topless to exhibit repression activity. However, further *in vivo* experiments are needed to confirm this hypothesis. Collectively, these data suggest *SRGs* may repress a repressor of defence-related genes, leading to the initiation of the immune response.

Additionally, the transcriptional profiling data revealed that oxidative- and immunity-related genes were enhanced in overexpressing-*SRG3* lines ([Davletova et al., 2005](#)). *WRKY70*, an important regulator of plant immunity ([Li et al., 2004](#), [Li et al., 2006](#)) and other environmental stresses ([Li et al., 2013](#)), was significantly

increased in *SRG2* and *SRG3* overexpression lines ([Rizhsky et al., 2004](#), [Ciftci-Yilmaz et al., 2007](#)). In our study, we also found that the key SA signalling marker gene, *pathogenesis-related 1 PR1*, was significantly increased in all *SRG-OX* lines (Figure 5.8). It is possible that overexpression of SRGs may repress the repressors which block the transcription of defence response genes such as *PR1* or *WRKY70*, thus leading to their transcriptional activation and the subsequent expression of disease resistance.

Based on our data, we have propose a hypothetical working model for *SRGs*-mediated signalling in plant immunity (Figure 7.1).

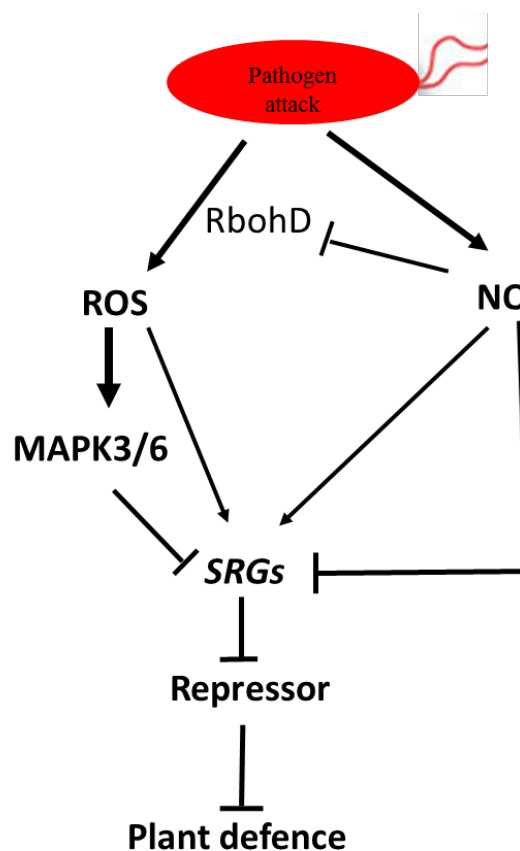


Figure 7.1 Preliminary model showing a key role of *SRGs* in the regulation of plant immunity.

Following pathogen challenge, ROS and NO levels increase and quickly activate expression of *SRGs*. Subsequently, *SRGs* might suppress the transcription of one or more unknown genes that encode repressor of defence-related genes, thereby, contributing to the activation of the plant immune response. Additionally, ROS can activate MAPK3/6, which may repress *SRGs* in the

posttranslational level, and NO may also repress SRGs in posttranslational level, suggesting regulation of SRGs in plant immunity is very complex and there may be some other machineries.

7.2 SRGs may function as an important regulator of NO-mediated transcriptome in plant immunity

NO activity is transferred by S-nitrosylation of the thiol group in active cysteine residues, regulating key physiological processes ([Wang et al., 2006](#)). In recent years, many key regulators of plant development and stress responses were identified as targets for S-nitrosylation ([Yu et al., 2014](#)). It has been proposed that NO is also capable of modulating gene expression ([Sha and Marshall, 2012](#)). However, little is known about how NO reprograms gene expression. Here, we found a sub-group of zinc finger containing transcription factors, SRGs, the transcripts of which accumulate in response to NO (Figure 3.5, 3.6 and 3.7). Further, we have shown that transcriptional activity of the SRG1 promoter can be activated by NO both *in vitro* and *in vivo* (Figure 4.7 and 6.2), leading to the accumulation of SRG1. Subsequently, SRG1 may repress the transcription of one or more genes that encode a repressor function that negatively regulates plant immunity. By extension, our results imply that SRG2 and SRG3 might function in a similar fashion.

The results presented here suggest that SRG1 might function as an important regulator of NO signalling. Modification of SRG1 by NO might modulate its ability to reprogramme the transcriptome in response to stress stimuli. Further, the cysteine residues in the zinc finger domain, a DNA binding motif, of this class of protein, have been regarded as a possible target of NO ([Kroncke et al., 1994](#), [Kröncke, 2001](#)). A number of lines of evidence provides indirect support for this hypothesis ([Serpa et al., 2007](#), [Sha and Marshall, 2012](#), [Okamoto et al., 2014](#)). In our study, we establish for the first time that NO directly modifies SRG1 by S-nitrosylation.

Interestingly, using the biotin switch assay (Figure 4.5), our data suggests that NO might selectively modify SRG proteins. Thus, SRG1 and SRG3 are S-nitrosylated, SRG2 is not, even though the cysteine target sites for this modification are highly conserved among these SRGs. This selectivity in SNO formation might be a

consequence of subtle differences in structure between SRG proteins reflecting the fact that proteins within the same family may exhibit different functions and thus may be regulated differently.

The activity of a transcription factor typically needs to be controlled in both time and space. We speculate that at the late stage of the immune response the activity of SRG1 can be inhibited by NO, following the accumulation of this redox active small molecule to a threshold value. Our data suggests this NO-mediated inhibition occurs through the S-nitrosylation of SRG1 at a reactive cysteine residue. SRG1-SNO formation appears to blunt SRG1 binding to its cognate *cis*-element. Thus, S-nitrosylation of this ZnTF may serve to inhibit its ability to repress the transcription of one or more genes encoding immune repressor functions. Consequently, the accumulation of these immune repressor proteins at a late stage of the immune response might contribute to a negative feedback loop leading to the cessation of defence signalling.

Interestingly, in addition to S-nitrosylation, SRGs might also be regulated by phosphorylation. In this context, SRGs possess a serine/threonine phosphorylation motif, which constitutes a possible phosphodegradon signal, and also a potential MAPK docking site. Further, SRG1 can interact with MAPK3/6 *in vitro and vivo* and significantly, SRG1 levels can be regulated by the 26S proteasome system (chapter 4). Collectively, these data suggest that SRG1 activity may also be regulated by phosphorylation modulated protein turnover, in addition to redox regulation.

Based on our data, we propose a potential working model (Figure 7.2) for SRG1 function in plant immunity.

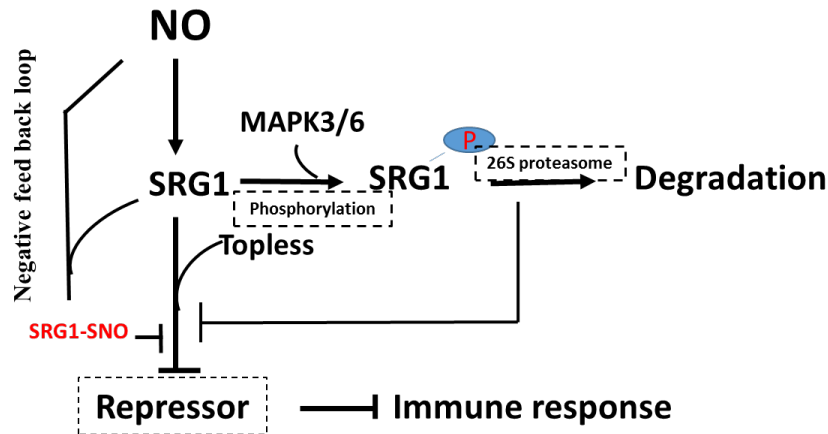


Figure 7.2 Proposed model suggesting the function of SRG1 in plant immunity.

Upon pathogen recognition, NO production induces *SRG1* expression. SRG1 can enhance the defence response by suppressing the accumulation of an unknown repressor of the defence response, leading to the activation of immune responses. At a later stage of the defence response, when SNO/NO levels reach a critical threshold level, SRG1 becomes S-nitrosylated. This primes a negative feedback loop to switch off the immune response. In parallel, phosphorylation of SRG1 by MAPK3/6 at later stages of the immune response results in SRG1 degradation via the 26S proteasome, which can repress SRG1 function.

7.3 Conclusion

In summary, we demonstrated some key points as follows:

- (1) SRGs are involved in plant immunity and act as positive regulators.
- (2) SRGs can interact with Topless and function as transcriptional repressors.
- (3) MAPK3/6 possibly interacts with SRG1/3 leading to phosphorylation.
- (4) NO can modify SRG1 DNA binding activity through S-nitrosylation.
- (5) SRGs may function as important regulators of NO-signalling in plant immunity.

7.4 Future prospects

Although we established a role of SRG1 in plant immunity, its exact mechanism of action remains unknown. To further expand knowledge of SRG1, several experiments could be performed in the future as followings:

- (1) The cysteine(s) modified by NO in SRG1 remains to be identified.

Identification of cysteine could be accomplished by MS after a biotin switch assay and site-directed mutagenesis experiment could be conducted to confirm the MS data if cysteine(s) are identified.

(2) Downstream target gene(s) of SRG1 is unknown. Candidates could be identified by RNA-seq and Chip-seq screening in *SRG1-OX#1*, *SRG1-OX#1gsnor1-3* and Col-0 plants. The selected candidate gene(s) level in Col-0, *SRG1-OX#1* and *SRG1-OX#1gsnor1-3* plants could be tested and confirmed RNA-seq and chip-seq screening.

(3) The effect of NO on SRG1 activity could be explained by transient repression activity assay *in vivo* and EMSA *in vitro*.

In summary, beased on these experiment it would give us a better understanding on SRG1 function in NO signalling during plant immunity.

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